Pharmacologic Apoptosis Modulation in Lymphocytes: a Novel Approach to Prevent Allograft Rejection and Induce Tolerance

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von

Pietro Ernesto Cippà

von

Bellinzona TI

Promotionskomitee
Prof. Dr. Michael O. Hengartner (Vorsitz)
Prof. Dr. Thomas Fehr (Leitung der Dissertation)
PD Dr. Martin F. Bachmann
Dr. Andrew Bushell
Prof. Dr. Rudolf P. Wüthrich

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Zusammenfassung

Die beste therapeutische Option für Patienten mit Organversagen ist häufig eine Transplantation. Um die immunologische Abstossung eines Organs aus einem genetisch nicht-identischen Spender zu verhindern, müssen transplantierte Patienten lebenslang eine immunsuppressive Therapie einnehmen. Derzeit verfügbare immunsuppressive Medikamente sind sehr wirksam gegen die akute Abstossung aber weniger effizient in der Prävention der chronischen Abstossung, und verursachen zum Teil schwerwiegende Nebenwirkungen. Neue Strategien zur Abstossungsprävention sind die Voraussetzung für eine verbesserte Lebenserwartung und Lebensqualität nach Organtransplantation. Die beste Lösung wäre die Induktion von immunologischer Toleranz, ein Zustand, in welchem das Immunsystem das Transplantat ohne Immunsuppression akzeptiert, aber auf pathogene Keime reagiert; verschiedene ungelöste Probleme verhindern aber eine breite klinische Anwendung von diesem Prinzip. Die Apoptose, eine Form von vorprogrammiertem Zelltod mit einer entscheidenden Funktion für die Gewebe-Homöostase in multizellulären Organismen, reguliert das Immunsystem und spielt eine kritische Rolle in der Erhaltung der Selbst-Toleranz. In dieser Studie untersuche ich die potentielle Rolle einer pharmakologischen Modulation der Apoptose als eine neue Strategie zur Abstossungsprävention und Toleranz-Induktion.

Kapitel 1 ist eine Einführung zur Transplantationsimmunologie und zu den klinischen und experimentellen Optionen für die Abstossungsprävention. **Kapitel 2** erklärt die Prinzipien der Apoptose, deren Rolle in der Regulation des Immunsystems und die Optionen für eine pharmakologische Modulation der Apoptose. Im **Kapitel 3** werden die Resultate der Experimente über die immunsuppressive Wirkung der pro-apoptotischen Substanz ABT-737 *in vitro* und in einem Haut-Transplantationsmodell in der Maus vorgestellt. Die Wirkung von ABT-737 *in vivo* war begrenzt, aber verstärkt in Kombination mit dem Calcineurin Hemmer Cyclosporin A. Im **Kapitel 4** werden der Synergismus mit Cyclosporin A und die immunomodulatorische Wirkung von ABT-737 weiter untersucht. Diese mechanistischen Studien waren die Basis für die Entwicklung eines neuen Protokolls zur Toleranzinduktion, wie beschrieben im **Kapitel 5**. Die Wirkung von ABT-737 auf Gedächtnis-Lymphozyten, ein wichtiges Hindernis für Toleranz in der Klinik wird im **Kapitel 6** untersucht. **Kapitel 7** fasst die immunmodulatorische Wirkung von ABT-737 und anderen pro-apoptotischen Substanzen zusammen. Im **Kapitel 8** diskutiere ich die mögliche Applikation von diesem neuen Prinzip im Bereich der Transplantation.

Summary

Transplantation is often the best therapeutic option for patients with end-stage organ failure. However, organ transplantation from a genetically not identical individual inevitably leads to immunological graft rejection, which must be prevented by lifelong immunosuppressive therapy. Currently available immunosuppressive drugs effectively prevent acute allograft rejection, but are less effective in the long-term and induce major side effects. Therefore, new strategies are required to improve survival and quality of life after transplantation. Induction of donor-specific tolerance, a state in which the immune system accepts the transplanted organ without immunosuppression and normally reacts to pathogens, would represent the ideal solution to this problem. However, several obstacles preclude the establishment of tolerance induction protocols clinical practice. Apoptosis, a form of programmed cell death with a fundamental role in the maintenance of tissue homeostasis in multicellular organisms, is critically involved in the regulation of the immune system and in the maintenance of self-tolerance. In this project, I investigated the potential role of a pharmacological modulation of the apoptosis pathway as a new strategy to prevent rejection and to induce tolerance after solid organ transplantation.

Chapter 1 provides an introduction to transplantation immunology and to clinical and experimental approaches to prevent allograft rejection. **Chapter 2** deals with apoptosis, its role as a regulator of the immune system and the available options to pharmacologically modulate it. **Chapter 3** describes the immunosuppressive effect of the pro-apoptotic drug ABT-737 *in vitro* and in a skin transplantation model. The immunosuppressive effect of ABT-737 as a single agent was limited, but markedly increased in combination with the calcineurin inhibitor cyclosporine A. **Chapter 4** analyzes more in details the synergistic effect of these two drugs and characterizes the immunomodulatory properties of ABT-737. The information obtained from these mechanistic studies was the basis for the development of a new therapeutic approach to induce immunological tolerance targeting the apoptosis pathway, as described in **chapter 5**. Finally, the effect of ABT-737 on memory lymphocytes, a major barrier to tolerance induction in humans, is described in **chapter 6**. In **chapter 7** I give an overview about the immuno-modulatory properties of ABT-737 and other pro-apoptotic drugs. **Chapter 8** discusses the potential role of these agents in transplantation.

Riassunto

Il trapianto è spesso la miglior opzione terapeutica per pazienti con un'insufficienza organica, ma obbliga il paziente ad assumere per tutta la vita una terapia immunosoppressiva al fine di prevenire il rigetto, un'inevitabile risposta del sistema immunitario dopo trapianto di un organo da un donatore geneticamente non identico. I medicamenti attualmente disponibili sono adatti alla prevenzione del rigetto acuto, ma meno efficaci sul lungo periodo e sono associati ad effetti collaterali anche gravi. Per questa ragione occorre sviluppare nuove strategie per migliorare la speranza e la qualità di vita in pazienti trapiantati. La migliore soluzione in tal senso è l'induzione di tolleranza immunologica verso il donatore, uno stato in cui il sistema immunitario accetta l'organo trapiantato senza immunosoppressione e reagisce in modo normale contro agenti patogeni. Tuttavia diversi ostacoli precludono l'applicazione di protocolli mirati all'induzione di tolleranza in ambito clinico. L'apoptosi, una forma di morte programmata di fondamentale importanza nel mantenimento dell'omeostasi tissutale in organismi multicellulari, gioca un ruolo determinante nella regolazione del sistema immunitario e nel mantenimento della auto-tolleranza. In questo progetto valuto l'opportunità di sfruttare una modulazione farmacologica dei meccanismi che regolano l'apoptosi quale nuovo approccio per prevenire il rigetto e per indurre tolleranza immunologica.

Il **capitolo 1** introduce i principi immunologici responsabili per il rigetto e le strategie cliniche e sperimentali per prevenirlo. L'apoptosi, il suo ruolo quale regolatore del sistema immunitario e le opzioni farmacologiche per modularne i meccanismi sono esposti nel **capitolo 2**. Nel **capitolo 3** sono descritti i risultati dello studio sulle proprietà immunosuppressive della sostanza pro-apoptotica ABT-737 *in vitro* e in un modello di trapianto di pelle nel topo, che mette in evidenza come l'effetto di tale medicamento sia fortemente potenziato se combinato con la ciclosporina. Questo sinergismo è ulteriormente analizzato nel **capitolo 4**. La caratterizzazione delle proprietà immuno-modulatrici di ABT-737 rappresentano lo spunto per lo sviluppo di un nuovo protocollo per l'induzione di tolleranza immunologica basato sulla modulazione della apoptosi, come discusso nel **capitolo 5**. Nel **capitolo 6** si prende in considerazione ABT-737 per l'inibizione di linfociti memoria, un ostacolo fondamentale per l'applicazione della tolleranza in ambito clinico. Nel **capitolo 7** riassumo le proprietà immuno-modulatrici di ABT-737 e altre sostanze pro-apoptotiche e nel **capitolo 8** ne discuto le possibili applicazioni nell'ambito della prevenzione del rigetto.

Abbreviations

APC	Antigen presenting cell
AP-1	Activator protein 1
ATG	Anti-thymocyte globulin
Bcl-2	B cell lymphoma 2
BM	Bone marrow
CD	Cluster of differentiation
CML	Cell-mediated lympholysis
CFSE	Carboxyfluorescein succinimidyl ester
CNI	Calcineurin inhibitor
CsA	Cyclosporine A
CTLA4	Cytotoxic T-lymphocyte antigen 4
DISC	Death-inducing signaling complex
DR	Death receptor
DST	Donor specific transfusion
ELISA	Enzyme-linked immunosorbent assay
DMSO	Dimethly sulfoxide
FACS	Fluorescence-activated cell sorting
FoxP3	Forkhead box P3
GITR	Glucocorticoid-induced TNF receptor family-related gene
GvHD	Graft versus host disease
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
IAP	Inhibitor of apoptosis
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL	Interleukin
LFA	Lymphocyte function-associated antigen
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
MMF	Mycophenolate mofetil
mTOR	Mammalian target of rapamycin
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor-κB
PI	Propidium iodide
PD-1	Programmed death 1
TCR	T cell receptor
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cell
XIAP	X-linked inhibitor of apoptosis

Section I: General introduction

Chapter 1: Transplantation immunology

The immune self

The immune system protects the body from pathogenic microorganisms and tumors. The first critical task to achieve this aim is the **recognition** of harmful agents. The immune system detects invading microorganisms by two complementary and sequential strategies (Murphy, Travers et al. 2008). First, the innate immune system reacts to danger signals and recognizes pathogens relying on a limited repertoire of molecular structures that are typically expressed by microorganisms, so called **pathogen-associated molecular patterns** (PAMPs). This initial discrimination between self and non-self is of ancient origin and allows a rapid identification of infectious agents. However, throughout evolution pathogens developed a variety of solutions to overcome this sophisticated but rather static barrier. Therefore, vertebrates developed a more sophisticated immunological recognition system: **antigen receptors** (B and T cell receptors) are generated by random rearrangement of gene segments resulting in a receptor repertoire that enables the adaptive immune system to virtually recognize any antigen that can be encountered in nature (Tonegawa 1976). This dynamic and plastic system inevitably leads to the generation of lymphocytes with affinity to self-antigens, which have to be suppressed to avoid the catastrophic situation of the immune system reacting against the host body (the *"horror autotoxicus"* described by Paul Ehrlich). Thus, discrimination of self and non-self is a fundamental function of the adaptive immune system.

1. Each lymphocyte bears a single type of receptor with a unique specificity
2. Receptor occupation leads to cell activation
3. The differentiated effector cells derived from activated lymphocyte will bear receptors of identical specificity as the parental cell
4. Lymphocytes bearing receptors specific for ubiquitous self molecules are deleted at an early stage and are therefore absent from the repertoire of mature lymphocytes

Tab. 1. Postulates of the clonal selection theory

Ray Owen's seminal study of dizygotic cattle twins (Owen 1945) and Peter Medawar's experiments in mice and chickens (Billingham, Brent et al. 1953) showed that exposure to foreign tissue during embryonic development results in specific immunological non-reactivity (tolerance) to donor antigens, indicating that discrimination of self and non-self by the adaptive immune system is an acquired state. The explanation of this central principle of adaptive immunity was proposed by Frank M. Burnet and is known as the **clonal selection theory** (Tab. 1) (Burnet 1959): self-reactive lymphocytes are deleted before they can mature by an active process, which eventually defines the antigen receptor repertoire. Importantly, according to this concept, **tolerance** can be defined as a "specific depression of the immune response induced by a previous exposure to the antigen"(Tauber 1994). Thus, the **immune self** is not genetically determined and can be considered as a dynamically evolving entity, influenced by the interaction of the immune system with the environment. Although additional factors are involved in the maintenance of self-tolerance (e.g. regulatory T cells (Tregs)), the general concept of the clonal selection theory has been proved right. The concept of a

dynamic immunological selfhood opens the opportunity to modulate the immune self to treat autoimmunity and to prevent allograft rejection.

Mechanisms of allograft rejection

Transplantation of living cells, tissues or organs is often the only or the best treatment for patients with organ failure. Nowadays kidney, liver, heart, lung, pancreas, Langerhans islets, skin, hematopoietic stem cells, small intestine and composite grafts are successfully transplanted in the clinic (Morris 2004; Sayegh and Carpenter 2004). Transplantation of human **kidney** is the most frequent procedure in this field (181 kidneys transplanted in Switzerland in 2010 among a total of 389 organs) (Swisstransplant 2010) and, although alternative modalities of treatment are available, represents the most effective therapy for advanced renal failure in terms of survival and quality of life (Simmons and Abress 1990; Port, Wolfe et al. 1993). However, transplantation from a genetically dissimilar individual (**allogeneic transplant**) can be considered as a iatrogenic violation of the immune self that induces an immune response in the host and inevitably leads to graft rejection (Nankivell and Alexander 2010).

Rejection of allografts is the result of a complex immune response involving the transplanted tissue and the host innate and the adaptive immune system (Fig. 1) (Morris and Knechtle 2008). The trauma caused by the surgical procedure and by the hemodynamic and neuroendocrine response to brainstem death in deceased donors leads to an inflammatory response in the transplanted tissue and activate the **innate immune system** (Meltzer, Veillette et al. 2012). This early antigen-independent response is not sufficient to induce rejection, as demonstrated by the acceptance of syngeneic grafts, but plays an important role in the stimulation of the subsequent antigen-specific immune reaction (Kim, Bedi et al. 2008). Activation of the endothelium and induction of cytokines, such as IL-6 and IL-1, promotes the graft infiltration of inflammatory cells and the migration of tissue-resident, bone-marrow-derived dendritic cells (or **passenger leukocytes**) from the transplanted organ to the host lymphoid tissue, where donor antigens are recognized by the adaptive immune system (Larsen, Morris et al. 1990; Celli, Albert et al. 2011).

The **major histocompatibility complex** (MHC; human leukocyte antigen – HLA – in humans) encodes for the most important tissue antigens in transplantation and plays a crucial role in determining survival of transplanted organs (Opelz and Wujciak 1994). In humans it is encoded on chromosome 6 and can be divided in three classes (Beck, Geraghty et al. 1999): MHC class I proteins are expressed on all nucleated cells and are responsible for the presentation of peptides primarily from the intracellular environment to CD8 T cells; MHC class II proteins are constitutively expressed on professional antigen presenting cells (such as dendritic cells, macrophages and B lymphocytes), but can be induced in many other cell types (such as renal tubular epithelial cells (Waeckerle-Men, Starke et al. 2007)) and are responsible for the presentation of peptides from the extracellular environment to CD4 T cells; the MHC class III region encodes for other proteins with disparate functions in the immune system and is less relevant in this particular context. At least six HLA loci are recognized in humans: the three class I genes (HLA-A, HLA-B, HLA-C) and the three main class II genes (HLA-DP, HLA-DQ, HLA-DR) are transmitted as a single mendelian trait in a maternal and a paternal haplotype and are codominantly expressed, so that siblings will have a 25% chance to be HLA-identical, 50% haploidentical and 25% full-mismatched. Importantly, MHC molecules are characterized by an extensive polymorphism (Gaur and Nepom 1996), so that MHC-identical unrelated individuals are very rare.

An additional barrier to transplantation is represented by **minor histocompatibility antigens**: polymorphic peptides presented by MHC molecules that assume a particular role in MHC-matched transplant combinations (Peugh, Superina et al. 1986). The best characterized example is the male-specific

HY antigen: females lack the Y chromosome and recognize peptides encoded by Y chromosome as foreign. Several other minor histocompatibility antigens have been described and directly reflect the intraspecies genetic variation, which assumes a renewed relevance after sequencing of the human genome. However, not all polymorphic pep-

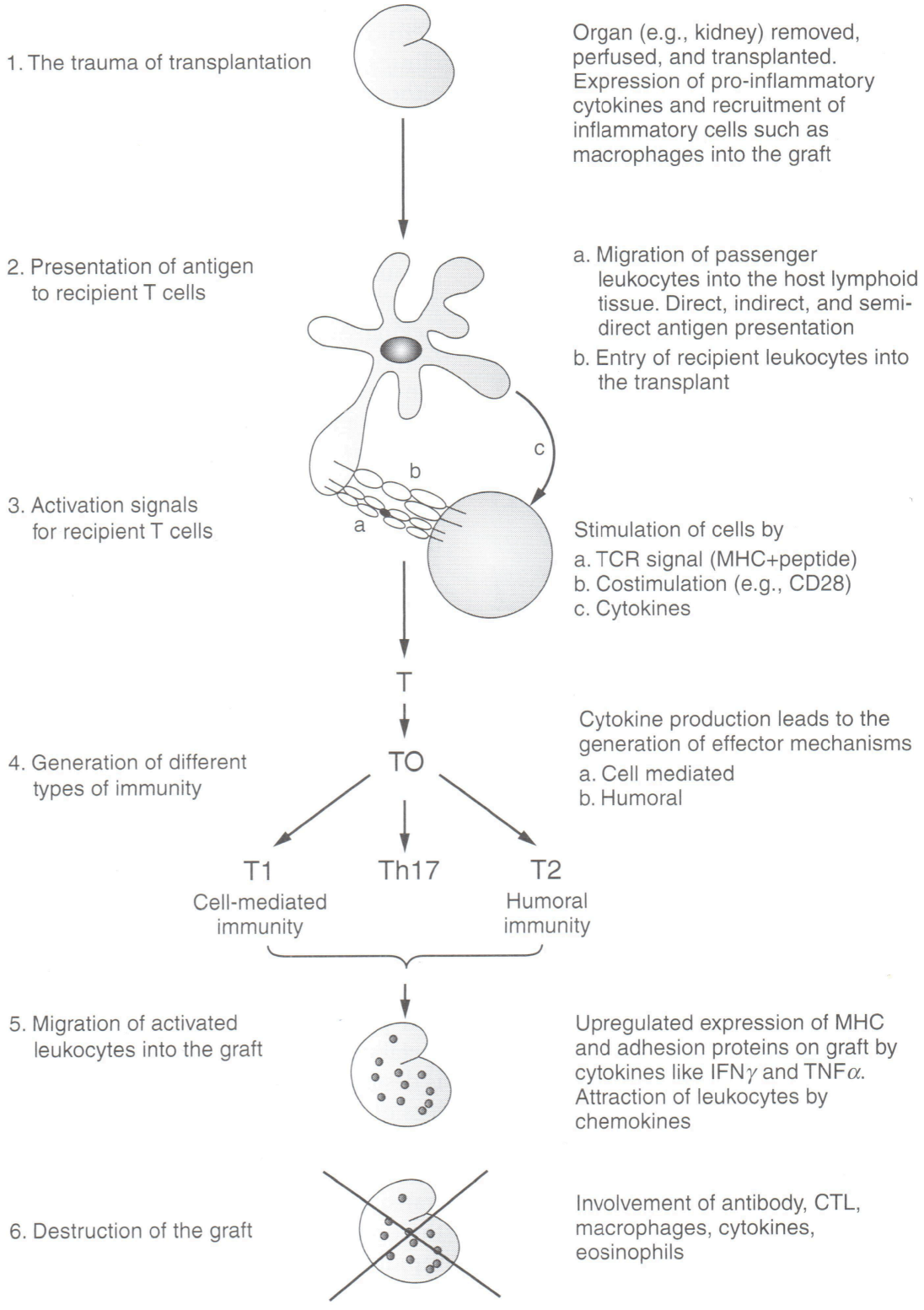

Fig. 1. The immune response after solid organ transplantation (Morris and Knechtle 2008)

tides display the same immunogenicity and only a limited number of them have been shown to play a relevant role as minor histocompatibility antigens (Roopenian, Choi et al. 2002). Thus, identity of histocompatibility antigens is in fact only possible in monozygotic twins, the only combination, in which organ transplantation will not result in graft rejection.

T lymphocytes are key players in most forms of acute rejection (Rosenberg and Singer 1992). As introduced above, the T cell repertoire is the result of positive and negative selection in the thymus: only T cells that express a T cell receptor (TCR) that binds to self-peptide/self-MHC complexes but do not react too strongly with self-antigens survive and migrate to peripheral lymphoid organs as mature naïve T cells. The aim of this process is the generation of a broad T cell repertoire to recognize all possible invading organisms with a minimal risk for autoimmunity. Thus, T cells bearing a TCR that recognize donor antigens (not expressed by the recipients) are not deleted from the repertoire and react to alloantigens in three different ways (Fig. 2) (Gould and Auchincloss 1999). **Direct allopresentation** occurs when a recipient T cell recognizes a donor-peptide/donor-MHC complex presented by donor passenger leukocytes migrating from the graft into the regional lymphoid organs in the initial phase after transplantation. Because T cells are positively selected to bind MHC molecules, the allogeneic peptide/MHC complex provides a strong stimulus to the immune system and a high percentage of T cells (1-10%) is typically activated after exposure to alloantigens (Reiser, Darnault et al. 2000; Nesic, Maric et al. 2002). For an **indirect allopresentation** donor-derived antigens are processed by antigen presenting cells of recipient origin and presented in the context of a donor-peptide/recipient-MHC complex. Because of their polymorphism, peptides from MHC molecules are critical in this setting, but minor histocompatibility antigens are presented in the same way. Cross-presentation (presentation of extracellular proteins on MHC class I) represents an alternative form of indirect

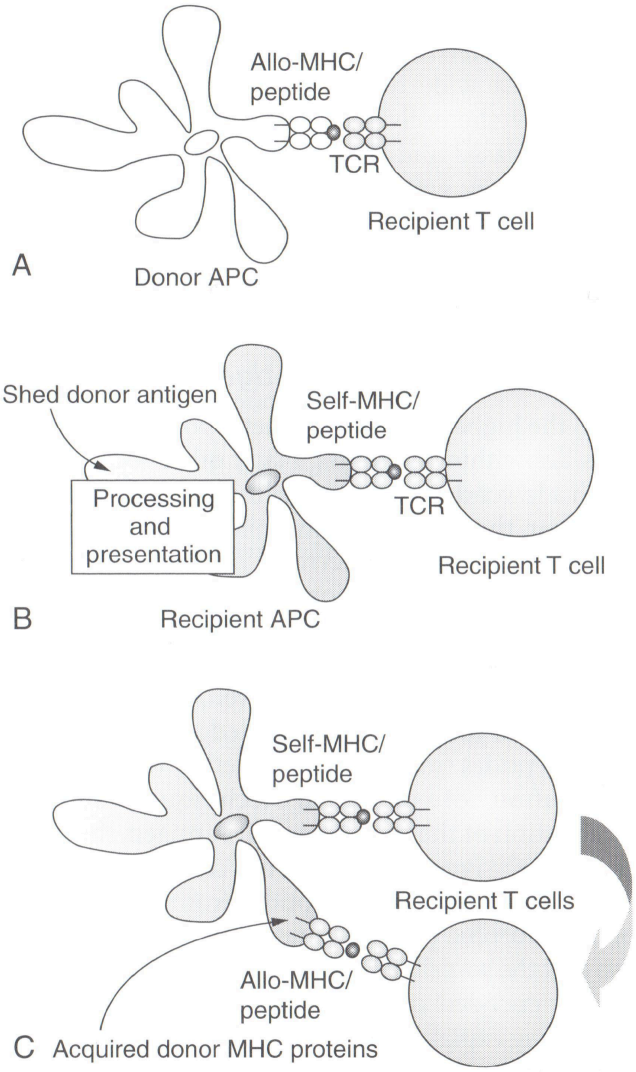

Fig. 2. Direct (A), indirect (B) and semidirect (C) allo-recognition (Morris and Knechtle 2008)

allopresentation (Carbone, Kurts et al. 1998). Moreover, it has been shown that recipient dendritic cells can acquire intact donor-MHC molecules. This process, called **semidirect allopresentation**, may explain how T cells that have been primed by recipient-derived dendritic cells can target the graft itself (expressing only donor-derived MHC), but the relevance of this mechanism remains to be assessed (Russo, Zhou et al. 2000).

Binding of the TCR is necessary but not sufficient for a full activation of a naïve T cell. Cell-adhesion molecules (such as CD2, LFA-1 and ICAM-3) are additionally required to stabilize the interaction between T cells and antigen presenting cells (APCs) in the su-

pramolecular organization of receptors and ligands called **immunological synapse** (Bromley, Burack et al. 2001). Moreover, according to the three-signal model, TCR triggering (signal 1) results in activation and differentiation of a naïve T cell only in combination with **co-stimulation** (signal 2) and a differentiation stimulus mostly provided by **cytokines** (signal 3) (Kapsenberg 2003; Halloran 2004). The most important costimulation is provided by the interaction of CD28 with B7 molecules and of CD154 (CD40L) with CD40, but these processes are known to be partially redundant. Moreover, additional co-stimulatory (such as CD27, TIM or 4-1BB) and co-inhibitory molecules (such as CTLA-4 or PD-1) influence signal 2 in a complex dynamic interaction (Clarkson and Sayegh 2005), which has assumed a therapeutical relevance in the field of transplantation in the last years (s. next section). In the presence of an adequate costimulation, T cells proliferate, differentiate into effector T cells and migrate into the graft.

The **destruction** of the graft is orchestrated by the differentiation of CD4 T cells in Th1, Th2, Th17 and Treg cells and is accomplished by a direct injury of transplanted tissue and by a further activation of endothelial and inflammatory cells. Cytotoxic CD8 T cells induce cell death in target cells by releasing perforin and granzymes and by a Fas-dependent activation of the extrinsic apoptosis pathway. CD4 T cells directly damage the graft by secreting tumor necrosis factor (TNF) α and β. Antibodies targeting donor MHC molecules, natural killer cells, macrophages, eosinophils and other cytokines are additionally involved in the complex process of allograft rejection (Nankivell and Alexander 2010).

The mechanisms described here typically occur in the context of an acute allograft rejection. Other mechanisms are involved in hyperacute and chronic rejection. **Hyperacute rejection** is mediated by preformed or natural antibodies targeting ABO blood group antigens, or MHC class I or class II molecules in sensitized recipients. Rejection occurs within minutes after revascularization through activation of the complement system. This problem can be avoided by ABO-matching and is largely a thing of the past since the introduction of pretransplant cross-match screening (Kissmeyer-Nielsen, Olsen et al. 1966). **Chronic allograft rejection** leads to a progressive deterioration of the graft function over years and has assumed a critical relevance since the success of modern immunosuppression to control acute rejection (s. next section). In the case of kidney transplantation, chronic allograft nephropathy is characterized by a concentric arteriosclerosis of graft blood vessels, with interstitial fibrosis and tubular atrophy. Immunological mechanisms, chronic inflammation, ischemia-reperfusion injury, endothelial dysfunction and pharmacological factors are likely to be involved in the pathogensis of this incompletely understood process (Bedi, Riella et al. 2010; Nankivell and Kuypers 2011).

Strategies to prevent allograft rejection

Preventing allograft rejection is the critical issue in transplantation medicine. The success of transplantation in the last decades is in large part attributable to the introduction of effective immunosuppressive drugs, mostly targeting the complex process of T cell activation and proliferation (Halloran 2004; Taylor, Watson et al. 2005). The most important classes of immunosuppressive drugs in clinical transplantation and their mechanisms of action are summarized in this section (Tab. 2, Fig. 3).

Glucocorticoids are an old class of drugs with multiple anti-inflammatory and immunomodulatory effects and are still routinely used in most immunosuppressive regimens and as a first-line treatment of acute rejection episodes. Steroids bind to the glucocorticoid receptor in the cytoplasm inducing its translocation into the nucleus. The interaction of the steroid-receptor complex with transcription factors such as activator protein 1 (AP1) and nuclear factor-κB (NF-κB) influences the transcription of a bunch of genes critically involved in allograft rejection (such as IL-1, IL-2, IL-6, IFN-γ and TNF-α). At

1. Glucocorticoids
2. Calcineurin inhibitors
   - Cyclosporine A
   - Tacrolimus
3. mTOR inhibitors
   - Sirolimus
   - Everolimus
4. Anti-proliferative drugs
   - Mycophenolate
   - Azathioprine
5. Depleting antibodies
   - Polyclonal horse or rabbit antithymocyte globulin (ATG)
   - Anti-CD3: Muromonab-CD3 (OKT-3)
   - Anti-CD52: Alemtuzumab (Campath-1H)
   - Anti-CD20: Rituximab
6. Non-depleting antibodies and fusion proteins
   - Anti-CD25: Basiliximab, Daclizumab
   - Anti-B7: Belatacept
7. Intravenous immune globulin

Tab. 2. Classification of immunosuppressive therapies used in organ transplantation

higher doses additional receptor-independent effects have been described (Adcock and Ito 2000).

Anti-proliferative drugs are the second important historical class of immunosuppressants (Elion 1993). Only with the combination of steroids and the anti-metabolite **azathioprine** in the early 1960s effective immunosuppression became a reality and the first allogeneic transplantations were successfully performed (Calne 1960; Murray, Merrill et al. 1963). Azathioprine is metabolized to 6-mercaptopurine and incorporated into DNA and RNA blocking replication and transcription. Moreover, it inhibits the *de novo* purine synthesis pathway with a partial selectivity for lymphocytes. As a result, lymphocyte proliferation and IL-2 production are markedly inhibited by azathioprine, but bone marrow depression inevitably occurs. Recent studies revealed that azathioprine additionally interferes with costimulation signaling, converting CD28 costimulation into an apoptotic stimulus (Tiede, Fritz et al. 2003). Anti-proliferative agents are still part of most immunosuppressive regimens but inhibitors of nucleotide synthesis are used instead of anti-metabolites in most centers, because of better efficacy and reduced toxicity. **Mycophenolate mofetil** (MMF) is the most important member of this class; it selectively suppresses purine synthesis in lymphocytes by inhibition of the enzyme inosine monophosphate dehydrogenase (Sollinger 1995).

Calcineurin inhibitors (CNIs) revolutionized transplantation in the 1980s and have represented the cornerstone of immunosuppression for more than two decades (Borel, Feurer et al. 1977). CNIs suppress T cell activation by inhibition of the TCR – calcineurin – nuclear factor of activated T cells (NFAT) pathway (signal 1) in the early phase after exposure to the alloantigen (Clipstone and Crabtree 1992). CNIs exert their immunosuppressive effect only after binding to immunophilins (more precisely, **cyclosporine A** (CsA) binds to cyclophilin A and **tacrolimus** (FK506) to FKBP-12); both complexes inhibit the phosphatase activity of calcineurin, preventing the de-phorphorylation and the nuclear translocation of NFAT. Blocking signal 1 dramatically reduces IL-2 production and IL-2 receptor (CD25) expression resulting in an abortive T cell activation. Several additional effects of CNIs have been described: CsA enhances the expression of transforming growth factor β (TGF-β), influences the regulation of apoptosis in lymphocytes (s. chapter 2) and the activation of dendritic cells (Chen, Guo et al. 2004). Moreover, CsA reduces the expression of MHC class II in renal epithelial cells and therefore the antigenicity of transplanted kidneys (Milton, Spencer et al. 1986). Unfortunately, immunophilins and calcineurin have been found to play a role in a variety of cell types other than lymphocytes, resulting in major side effects (Kiani, Rao et al. 2000). Therefore, more selective NFAT-inhibitors have recently been developed to block signal 1 without calcineurin inhibition (Aramburu, Yaffe et al. 1999).

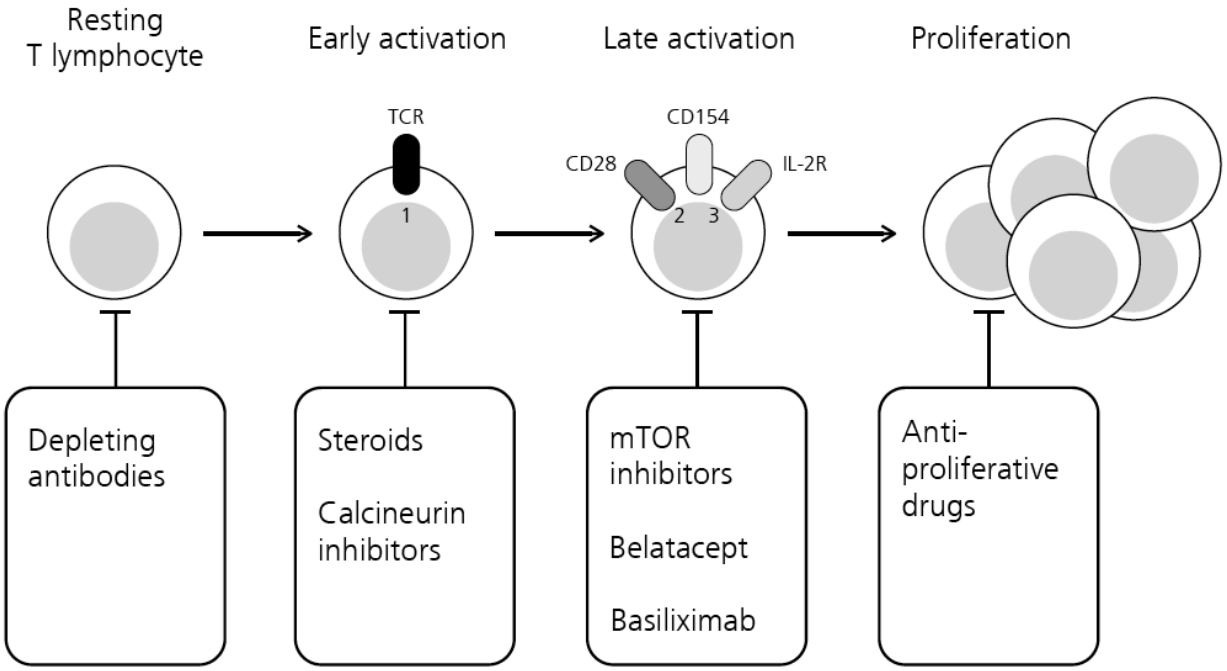

Fig. 3. T cell activation and site of action of immunosuppressive drugs

Similar to FK506, **mTOR inhibitors** also bind to the immunophilin FKBP-12, but exert their immunosuppressive effect by inhibiting the mammalian target of rapamycin (mTOR), a key component of the cell cycle regulatory signaling (Heitman, Movva et al. 1991). In lymphocytes, the stimulus for proliferation is provided by triggering cell surface receptors (particularly by binding of IL-2 to IL-2R), which leads to activation of janus kinase 3 and mTOR complex 1 (signal 3). The FKBP-12-sirolimus complex blocks this process, preventing proliferation of activated T cells. **Sirolimus** and **everolimus** are mostly used in CNI-free immunosuppression regimens in combination with MMF and may have a clinically relevant anti-neoplastic effect (Webster, Lee et al. 2006).

The small molecule immunosuppressive drugs briefly described here are often combined with protein drugs (biologics). **Antibody** preparations, usually classified as depleting or nondepleting, exert their immunomodulatory effect in different ways. The most important mechanisms are complement-dependent lysis of targeted cells, mimicking of the physiological ligand resulting in triggering or blockade of signal transduction, induction of surface molecule internalization and stimulation of phagocytic cells (Morris and Knechtle 2008). Techniques to generate antibodies with a single, genetically defined monoclonal specificity opened the opportunity to target surface molecules involved in adaptive immune responses and therefore to establish selective therapeutic options. This led to an explosion in the development of biologics in the field of transplantation and autoimmunity in the last years. Polyclonal (anti-thymocyte globulin – ATG) and monoclonal (muronomab-CD3, alemtuzumab, rituximab) lymphocyte **depleting** antibodies are generally indicated for induction therapy and for the treatment of refractory rejection. Nondepleting monoclonal antibodies and fusion proteins have been designed to bind to essential factors in the activation of alloreactive T cells. Blockade of signal 2 is a promising approach in this setting and the B7-directed fusion protein **belatacept** has recently been shown to provide a good immunosuppression after kidney transplantation without the nephrotoxic side effects of CNIs (Vincenti, Larsen et al. 2005). Inhibition of CD40-CD154 signaling by targeting CD154 showed great prom-

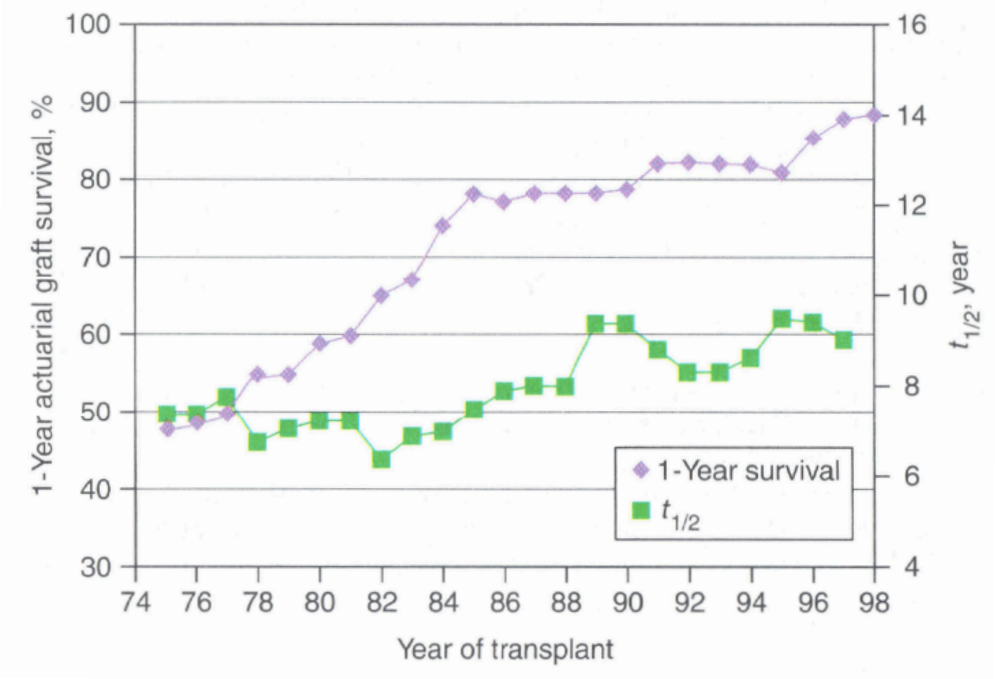

Fig. 4. One-year graft survival and graft half-life in a cohort of first deceased kidney transplant recipients (Kasper and Harrison 2005)

ise in rodent models (Larsen, Elwood et al. 1996), but first clinical trials failed because of unexpected thromboembolic side effects (Kawai, Andrews et al. 2000). Signal 3 can be inhibited by anti-CD25 antibodies (**daclizumab** and **basiliximab**). These antibodies bind to the IL-2R and hinder the activation of the receptor by IL-2, and may have additional beneficial effects on Tregs (Vincenti, Kirkman et al. 1998).

Thus, a diversified armamentarium of drugs is currently available to prevent and to treat acute allograft rejection. The progressive development of novel immunosuppressive strategies was accompanied by miraculous outcome improvement (Morris 2004). After the initial discouraging experiences, the introduction of azathioprine in combination with steroids allowed to achieve a 1-year graft survival of 50% in the late 1970s. But it was the discovery of CNIs that radically changed the efficacy of immunosuppression, leading to a continuous improvement in 1-year graft survival (Fig. 4). Current standard immunosuppressive regimes, including CNI, MMF and steroids, in combination with an induction therapy with depleting or anti-CD25 antibodies for patients with a high risk for rejection, result in **95% 1-year graft survival** in most centres (Hariharan, Johnson et al. 2000; Ekberg, Tedesco-Silva et al. 2007). Thus, nowadays acute allograft rejection after solid organ transplantation is a preventable and curable disease, but major problems remain to be solved.

Currently available immunosuppressive drugs unselectively inhibit the immune system and induce a **generalized immunosuppression**. Therefore, the ameliorated efficacy in allograft rejection was inevitably related to a risk for infections and tumors (Fishman and Rubin 1998; Kasiske, Snyder et al. 2004). The identification of immunological markers to estimate the degree of immunosuppression required by each single patient would allow to better balance the trade-off between prevention of allograft rejection and over-immunosuppression (Cippa and Fehr 2011). Moreover, the long-term exposure to these drugs results in major side effects not directly related to their immunosuppressive activity. Importantly, several immunosuppressive drugs have a negative impact on **cardiovascular risk** factors: dyslipidemia is a common side effect of CNIs, mTOR inhibitors and steroids, hyper-

tension and diabetes are often registered in patients treated with CNIs and steroids (Feehally, Floege et al. 2007). These factors do not directly influence 1-year graft survival but have a deleterious long-term impact on patient survival and quality of life. Moreover, the well-known **nephrotoxic** properties of CNIs lead to a progressive deterioration of renal function after transplantation (Cippa and Fehr 2010). Thus, about 50% of the renal graft loss beyond the first year after transplantation is due to the death of the patient with a functioning graft, mostly because of cardiovascular events (Pascual, Theruvath et al. 2002). Moreover, despite their potency against acute rejection, currently available immunosuppressive drugs are much less effective in preventing **chronic allograft rejection** (Nankivell and Kuypers 2011). As a result, the long-term outcome after transplantation could not significantly be improved in the last decades (Fig. 4) and innovative strategies are required to approach this challenging issue.

Two principles have to be pursued for the establishment of new strategies for allograft rejection control: **tissue-selectivity** and **allo-selectivity**. First, to avoid off-target side effects, immunomodulatory drugs or procedures should selectively target the tissues involved in allograft rejection. Considering the critical role of the adaptive immune system in this setting, the lymphatic cells are the most obvious target, but the transplanted organ may represent an additional element of interest as trigger and as final target of the immune response (Cypel, Yeung et al. 2011). Second, to avoid generalized immunosuppression, only allo-reactive lymphocytes should be suppressed in their activity. This task is particularly challenging: antigen-selectivity is determined by the antigen receptor (B and T cell receptor), but because of the high variability of this single selectivity marker, this does not represent a reasonable pharmacological target. Thus, allo-selectivity can probably only be achieved by exposing the recipient to donor antigens and modulating the properties of the responding lymphocytes. Exposure to donor antigens by the administration of a **donor specific transfusion** (DST) before transplantation has been shown to generate Tregs and may improve outcome of kidney transplants, but has been abandoned in most centers because the procedure induced sensitization to the donor in a subgroup of patients (Bushell, Karim et al. 2003; Marti, Henschkowski et al. 2006). However, this approach may assume a new relevance in combination with novel immunomodulatory agents. The molecular understanding of the T cell activation process is critical in this setting and the rational development of drugs binding to receptors selectively involved in T cell activation (such as costimulation molecules and CD25) is an important step in this direction. However, allo-selectivity will be inevitably lost if the immunomodulatory drug is administered long-term after transplantation, because the immune response against newly encountered environmental antigens would also be affected. Thus, the most promising approach to induce true allo-selectivity is represented by a short conditioning protocol including exposure to donor antigens that leads to a sustained donor-specific hyporesponsiveness or tolerance.

Transplantation tolerance

Induction of donor-specific tolerance, a state in which the immune system does not react against the transplanted tissue but normally responds to foreign antigens, conceptually corresponds to a **re-programming** of the adaptive immune system with a partial redefinition of the immune self (Waldmann, Adams et al. 2008). The first step towards the realization of this aim was taken in the 1950s by Peter Medawar, who demonstrated that tolerance is **acquired** during embryonic development in mice (Billingham, Brent et al. 1953). The early attempts to translate these results into clinical practice were mostly abandoned after the introduction of effective immunosuppressive drugs (Schwartz and Dameshek 1959; Murray, Merrill et al. 1960; Starzl and Zinkernagel 2001). However, the more recent recognition of the substantial limitations of immunosuppression in the long-term encouraged

	Mechanism	Site of action
Central clonal deletion	Apoptosis	Thymus, bone marrow
Peripheral anergy	Cellular inactivation by weak signaling without costimulation	Secondary lymphoid organs
Peripheral clonal deletion	Apoptosis	Secondary lymphoid organs and sites of inflammation
Regulatory cells	Suppression by cytokines, intercellular signals	Secondary lymphoid organs and sites of inflammation
Antigen segregation	Physical barrier to antigen access to lymphoid system	Peripheral organs

Tab. 3. Mechanisms controlling self-tolerance (Murphy, Travers et al. 2008, modified)

the transplant community to reconsider allograft tolerance as a realistic aim in clinical transplantation (Fehr and Sykes 2004; Waldmann 2010; Dolgin 2012).

To find a way to establish tolerance in transplantation it is necessary to understand the mechanisms physiologically determining **tolerance to self-antigens**. This is the result of the concerted action of processes that operate at different sites and stages of development (Goodnow, Sprent et al. 2005; Murphy, Travers et al. 2008) (Tab. 3). As discussed before, **central clonal selection** is the first and most important checkpoint to eliminate newly formed auto-reactive lymphocytes, but central tolerization is not perfect and additional peripheral mechanisms are required to prevent autoimmunity.

Critical in this setting is the role of antigen presenting cells (APCs). "Danger signals" delivered by pathogens, by the innate and the adaptive immune system and by the tissue influence the activation (or "**licensing**") of APCs by regulating the expression of costimulatory and co-inhibitory factors. The "licensed" APC expresses high levels of MHC and costimulatory molecules such as CD40, CD80 and CD86 and fully activates T cells reacting against the presented antigens. In contrast, in the absence of adequate costimulation, reactive T cells become **anergic** and undergo **apoptosis**. Thus, T cells that escape central selection are inactivated or deleted from the repertoire when antigen recognition occurs in a "danger-free" environment.

Immunological **regulation**, resulting from a complex interaction between immune cells, is an additional critical mechanism for the maintenance of self-tolerance. It has been recognized that a population of CD4+CD25+FoxP3+ T cells, called regulatory T cells (Tregs), plays a critical role in this setting. This is well demonstrated by the induction of catastrophic systemic autoimmunity after selective ablation of FoxP3 expressing cells or their functional inhibition using an anti-GITR antibody (Sakaguchi, Sakaguchi et al. 1995; Shimizu, Yamazaki et al. 2002; Kim, Rasmussen et al. 2007). The majority of Tregs develops in the thymus (natural Tregs), but naïve CD4 T cells can also convert to Tregs (induced Tregs) when stimulated under the effect of TGF-β and IL-10 (Chen, Jin et al. 2003). Moreover, differentiation to Tregs can be obtained experimentally by activation of naïve CD4 T cells under the effect of rapamycin (Battaglia, Stabilini et al. 2005), anti-CD154 (Taylor, Friedman et al. 2002) or phosphodiesterase inhibitors (Feng, Nadig et al. 2011). Tregs exert their immunosuppressive effect influencing other lym-

phocytes and APCs via direct cell-cell contact, secretion of interleukins or by cytotoxicity (Wood, Bushell et al. 2011). Moreover, Tregs promote the generation of additional Tregs, thereby sustaining peripheral tolerance over time in a process called **infectious tolerance** (Qin, Cobbold et al. 1993).
In the last years, there was an increasing interest on the role of **peripheral tissues** in the regulation of the adaptive immune system (Matzinger and Kamala 2011). Since decades it has been known that tissue grafts transplanted in so-called **immune privileged sites** (such as the anterior eye chamber or the testes) are not rejected by the immune system (Medawar 1948). Physical barriers that protect the tissue from an interaction with the immune system have been described in these particular parts of the body, but additional regulatory mechanisms are likely to be involved. Similar mechanisms probably explain the lack of rejection of the mammalian fetus by the maternal immune system, the only example of allogeneic tolerance physiologically occurring in nature (Mold, Michaelsson et al. 2008).

The processes involved in the maintenance of self-tolerance briefly discussed here can be exploited to establish experimental strategies to induce donor-specific tolerance aiming at acceptance of solid organ transplants.
Central tolerance can be achieved through the induction of **mixed chimerism** and is discussed in details in the next paragraph.
Peripheral anergy and clonal deletion are obtained by **costimulation blockade**. A combined costimulation blockade using the anti-CD154 (anti-CD40L) antibody MR1 and CTLA4Ig was sufficient to induce long-term acceptance of cardiac or islet allografts in mice (Larsen, Elwood et al. 1996), but not in more stringent models of skin grafting or in large animals (Markees, Phillips et al. 1997; Kirk, Burkly et al. 1999). Moreover, a generalized allo-antigen exposure (as obtained with DST) under the cover of MR1 resulted in a substantial prolongation of MHC-mismatched skin graft survival and to permanent allograft acceptance in thymectomized mice, indicating that this procedure was sufficient to tolerize the peripheral immune system in naïve mice, but that newly arising T cells were not inhibited using this approach (Markees, Phillips et al. 1998).
Regulatory mechanisms are key elements in the modulation of the adaptive immune system, and **Tregs** may find a clinical application to induce tolerance in the near future (Leslie 2011). Two general strategies are pursued: the application of Tregs as a component of a cellular therapy or the development of drugs selectively modulating Tregs *in vivo* (Li and Turka 2010). Transfer of *ex vivo* generated or expanded CD4+CD25+FoxP3+ Tregs can prevent acute and chronic allograft rejection and development of transplant arteriosclerosis in mouse and humanized mouse models (Feng, Wood et al. 2008; Nadig, Wieckiewicz et al. 2010; Warnecke, Feng et al. 2010). However, Tregs are less effective in the inhibition of memory cells and T-effector cells activated under inflammatory conditions. Moreover, their unfavorable *ex vivo* expansion properties and technical limitations related to the high numbers of Tregs required for immune regulation remain to be solved for a clinical application of this approach. The selective stimulation of Tregs *in vivo* may represent a technically less challenging strategy. Antigen recognition under costimulation blockade or rapamycin generates allo-reactive Tregs that can induce tolerance in minor histocompatibility mismatched models (Graca, Honey et al. 2000; Lee, Rusche et al. 2001). Apart from CD4+CD25+FoxP3+ Tregs, **non-lymphoid regulatory cells**, such as regulatory dendritic cells (Steinman, Hawiger et al. 2003), mesenchymal stem cells (English and Mahon 2011) and regulatory macrophages are considered potential targets for the induction of tolerance (Dugast and Vanhove 2009).
Antigen segregation has also been investigated as a possible strategy to prevent allograft rejection. The most important application of this approach is the encapsulation of pancreas islets (Vaithilingam and Tuch 2011).

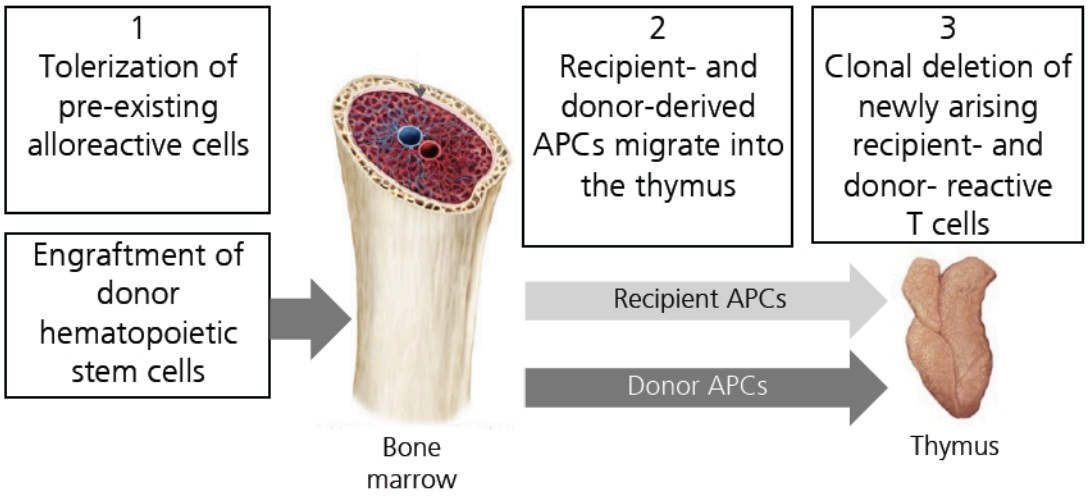

Fig. 5. Mechanism of tolerance in mixed chimeras

Mixed chimerism

It has been known for many years that transplantation of hematopoietic stem cells (HSCs) during development results in donor-specific tolerance (Billingham, Brent et al. 1953). This process is mediated by donor-derived APCs that migrate into the **thymus** and contribute to negative selection leading to a selective deletion of donor-reactive newly arising T cells. Obtaining the same result in a recipient with a mature immune system is much more difficult, because suppression of peripheral and intra-thymic alloreactivity is necessary. The most simple but invasive approach to achieve this aim in mice is a complete deletion of the preexisting immune system by lethal irradiation, followed by reconstitution with donor bone marrow (BM) to induce "**full chimerism**". This procedure is not clinically applicable because of the toxicity of the induction regimen and because of the high risk for **graft versus host disease** (GvHD) (Sykes 2001; Pilat and Wekerle 2010). However, it has been recognized that reconstitution with a mixture of donor and recipient BM to induce a state called **mixed chimerism**, in which recipient and donor HSCs co-exist, similarly leads to donor-specific tolerance (Ildstad and Sachs 1984). In this situation, recipient- and donor-derived APCs migrate into the thymus and induce **central deletion** of both recipient- and donor-reactive T cells (Fig. 5). Central tolerance in mixed chimeras is systemic and robust, as shown by acceptance of fully MHC-mismatched skin and vascularized allografts in rodents and large animals (Sachs, Sykes et al. 2011). Importantly, the induction of mixed chimerism is much less problematic than a full donor chimerism. First, because BM-derived cells are more efficient than thymic stromal cells for negative selection, the presence of recipient-derived APCs in the thymus is associated with a reduced risk for GvHD. Second, because deletion of recipient BM is not required, mixed chimerism can be achieved using less toxic non-myeloablative conditioning protocols (Sykes 2001).

The prerequisites to achieve mixed chimerism are a tolerization of the preexisting immune system and the engraftment of donor HSCs. A general re-setting of the peripheral adaptive immune system in naïve mice can be achieved by depleting doses of **anti-CD4 and anti-CD8** monoclonal antibodies, but the efficacy of this approach is limited in humans (Sykes, Szot et al. 1997). A more attractive strategy is represented by a selective inactivation or deletion of allo-reactive T cells using the tolerance induction strategies introduced before. This leads to a combined approach: a tolerance inducting therapy (effective in the short-term but not sufficient to maintain long-term tolerance by itself) allows engraftment of HSCs and the progressive establishment of central deletion maintains permanent tolerance.

The most investigated approach is the combination of HSC transplantation with **costimulation blockade**. In fact, a single injection of anti-CD154 (MR1) in combination with low-dose total body irradiation (TBI, 3 Gy) or donor specific transfusion (DST) was sufficient to induce mixed chimerism using standard doses of BM cells (20-50x10^6 cells/mouse) (Wekerle, Sayegh et al. 1998; Seung, Mordes et al. 2003). Double costimulation blockade with MR1 and CTLA4Ig induced mixed chimerism even without any cytoreductive treatment after injection of mega-doses of BM cells (200x10^6 cells/mouse) (Wekerle, Kurtz et al. 2000). The mechanism of **initial tolerance** in animals receiving BM cells under the effect of costimulation blockers is complex and not completely understood (Wekerle, Kurtz et al. 2002). After a phase of T cell anergy (Kurtz, Shaffer et al. 2004), the most important mechanism is **peripheral clonal deletion** of donor-reactive T cells (Wekerle, Sayegh et al. 1998). MR1-induced T cell deletion is the result of an incomplete APC licensing determined by the inhibition of the CD40-signaling and not a direct effect of CD154 binding on activated T cells (Kurtz, Ito et al. 2001). Incompletely activated APCs induce **apoptosis** in donor-reactive T cells primarily through activation of the intrinsic apoptosis pathway (s. chapter 2), but several studies indicated that an initial additional reduction in the number of donor-reactive T cells is required to establish tolerance across MHC barriers (Li, Li et al. 1999; Wells, Li et al. 1999; Li, Strom et al. 2001; Wekerle, Kurtz et al. 2001). Interestingly, although Tregs are generally implicated in costimulation-based protocols, in mixed chimerism models no evidence for classical regulatory T cells was detected (Kurtz, Shaffer et al. 2004; Kurtz, Wekerle et al. 2004). However, deletion of CD4 T cells (but not of CD25 cells) in the initial phase precluded mixed chimerism induction, demonstrating that the initial presence of CD4+CD25- cells is required for the tolerization of CD8 T cells (Fehr, Takeuchi et al. 2005; Fehr, Haspot et al. 2008). Although Tregs are probably not pivotal for mixed chimerism induction using non-ablative irradiation and MR1, it has recently been demonstrated that injection of *ex vivo* generated **Tregs** promotes mixed chimerism induction allowing tolerance induction without cytoreductive conditioning (Pilat, Baranyi et al. 2010). Further studies using this approach in non-human primates are ongoing.

In the first studies using the mixed chimerism approach, irradiation was systematically included in the conditioning protocol not only to suppress peripheral allo-reactive lymphocytes, but also to promote **HSC engraftment** with the assumption that a partial depletion of recipient hematopoietic stem cells was required to "create space" in the BM (Tomita, Sachs et al. 1994). The development of irradiation-free, non-myelosuppressive protocols demonstrated that this theory was wrong (Sykes, Szot et al. 1997; Wekerle, Kurtz et al. 2000). The mechanisms involved in BM engraftment after intra-venous injection of HSCs have only been partially elucidated. It is known that HSCs physiologically migrate from the BM to the blood (mobilization) and from the blood to the BM (homing) in normal animals. HSCs injected into the blood participate to this dynamic process and compete with circulating host HSCs for the available **stem cell niches** in the BM (Kondo, Wagers et al. 2003). Irradiation or cytotoxic agents may promote the mobilization of HSCs and facilitate the engraftment of donor derived HSCs, but this is apparently not necessary if mega-doses of BM are used (Wekerle, Kurtz et al. 2000). The adaptive immune system may play an additional role in engraftment and maintenance of allogeneic HSCs. Recent studies demonstrated that HSC niches in the BM are immune privileged sites (Fujisaki, Wu et al. 2011), so that the acceptance of donor HSCs may be maintained through local regulatory mechanism even before central deletional tolerance has been established.

Engraftment of donor APCs in the thymus is required to induce central tolerance (Fig. 5). Since monoclonal antibodies are not very efficient in depleting allo-reactive thymocytes, **thymic irradiation** was introduced to inhibit residual intra-thymic alloreactivity in

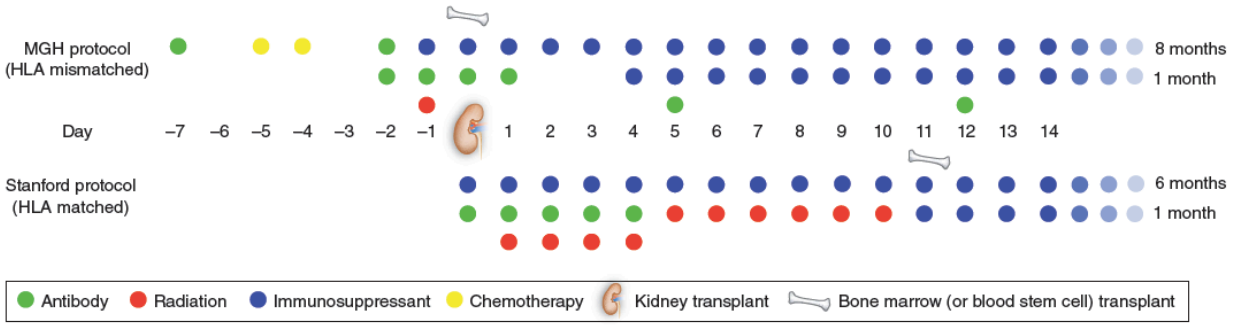

Fig. 6. Clinical protocols to induce mixed chimerism (Dolgin 2012)

non-myeloablative protocols (Sykes 2001). In rodents this could be replaced by the introduction of costimulation blockers or by a short course of CsA (Nikolic, Zhao et al. 2000), but thymic irradiation is still included in mixed chimerism induction protocols in clinical evaluation.

Among the different approaches to induce tolerance in rodents very few were successful in large animals and only the mixed chimerism model was translated into the first **pilot studies in patients**, principally by two independent groups in the United States (Fig. 6) (Fehr and Sykes 2008). The group of Samuel Strober at **Stanford** established a mixed chimerism induction protocol based on T cell depletion with anti-thymocyte globulin (ATG) and irradiation of lymphoid organs first in dogs and non-human primates and finally in kidney transplant patients (Strober, Modry et al. 1984; Myburgh, Smit et al. 2001). In the first clinical study with HLA-mismatched cadaveric kidney allografts only 3 of 28 patients developed tolerance and only one patient has been reported to be tolerant for more then 10 years (Strober, Dhillon et al. 1989; Strober, Benike et al. 2000). The recently reported results of a second trial using an intensified protocol and HLA-matched kidney allografts were much more encouraging with 8 out of 12 patients that had gone off of immunosuppresants for more then one year without complications (Scandling, Busque et al. 2011).

The group at **Massachusetts General Hospital** (David Sachs, Megan Sykes) was involved in the initial studies using the mixed chimerism approach in rodents and further developed it in pigs and non-human primates to establish a clinical protocol consisting of T cell depletion using ATG or a depleting monoclonal anti-CD2 antibody, cyclophosphamide, a short course of CsA and thymic irradiation (Sachs, Sykes et al. 2011). The first clinical study was performed in patients with renal failure secondary to **multiple myeloma**. As allogeneic BM transplantation is the only known cure for multiple myeloma, but conventional BM transplantation was contraindicated in these patients because of renal failure, a combination of BM and renal transplantation in a HLA-matched combination was considered the ideal option in this particular situation. Only one of the 9 patients treated with this protocol developed acute allograft rejection after discontinuation of the immunosuppressive therapy, and only 3 patients presented a recurrence on the myeloma (Fudaba, Spitzer et al. 2006). The second study was performed with **HLA-mismatched** renal allograft in patients without complicating neoplasia. In 8 of 10 patients immunosuppression has been discontinued and in 7 of them renal allograft function has been stable for a follow-up of 10 to 90 months (Kawai, Cosimi et al. 2008; Kawai, Sachs et al. 2011).

Despite the small numbers of patients and the partially unsatisfactory results, these pioneering studies demonstrated that tolerance induction through mixed chimerism is feasible in the clinic and highlighted several **limitations** that remain to be addressed for a broad application of this approach. The conditioning protocols required to induce mixed chimerism in patients are characterized by major **toxicity**, related to the fact that mixed chimerism induction in large-animals is much more difficult than in rodents, due to of immunological and pharmacological differences (Sykes 2001). Among the immunological factors, **memory cells** have been shown to preclude tolerance induction particularly in costimulation-based protocols in mice and non-human primates (Nadazdin, Boskovic et al. 2011). Interestingly, memory cells generated as a result of previous infections can crossreact with allo-antigens in a process called **heterologous immunity** (Adams, Williams et al. 2003) and provide a major barrier for the translation to the clinic of protocols established in laboratory mice that have been barely exposed to pathogens during their life (Ford and Larsen 2011). Pharmacological challenges are primarily related to the **thromboembolic side effects** registered in non-human primates and in humans after treatment with anti-CD154 (Kawai, Andrews et al. 2000) and to major differences in the response to lymphocyte depleting therapies among species: particularly, complete **T cell depletion** is possible in mice but is not realistic in humans (Sachs, Sykes et al. 2011). For these reasons less sophisticated but much more toxic regimes were chosen for the clinical studies, resulting in severe morbidity. All recipients developed **pancytopenia** and most of them **engraftment syndrome**, a systemic inflammatory response syndrome (SIRS) accompanied by fever, fluid retention and transient renal failure.

Moreover, although a systemic donor-specific tolerance persisted, non-human primates and patients **lost chimerism** during the first days after transplantation. Previous studies demonstrated that chimerism is needed only in the thymus to maintain tolerance in mice. However, thymic APCs are continually turning over and true hematopoietic chimersm is required to replace donor APCs over time in mice (Khan, Tomita et al. 1996). Thus, acceptance of the kidney graft and simultaneous rejection of the BM, determining a state of "**split tolerance**", occurred only in primates and may represent a substantial difference between species in this setting (Sachs 2003). Studies in monkeys indicate that the mechanism of tolerance may switch from central deletion to a peripheral mechanism that may include Tregs and in which the renal allograft plays a critical role (Andreola, Chittenden et al. 2011; Sachs, Sykes et al. 2011). A similar role of the allograft in the maintenance of regulatory mechanism has been previously described in a different mouse model (Karim, Steger et al. 2002).

An additional issue is provided by the combination of immunosuppressive and tolerogenic therapies. Because of the excellent graft survival obtained with classical **immunosuppression**, the risk of allograft rejection cannot be ethically accepted in the modern transplantation era and every tolerance-inductive therapy should initially be combined with immunosuppression in clinical studies (Waldmann 2010). However, immunosuppressive drugs can radically alter the outcome of the procedure, because of pharmacological and immunological interactions. Particularly, CNIs displayed an anti-tolerogenic effect in costimulation-based protocols (Blaha, Bigenzahn et al. 2003).

In summary, the induction of donor-specific tolerance is the best option to improve the long-term outcome after solid organ transplantation. Among the different strategies to induce tolerance in rodents, the combined transplantation of solid organ and BM from the same donor to induce mixed chimerism is the only approach that was successfully translated to the clinic, but several problems remain to be solved for its broad clinical application. The introduction of costimulation blockers allowed to markedly reduce the toxicity of the conditioning re-

gime in mouse models, changing the strategy for the tolerization of the pre-exisitng immune system from a generalized T cell depletion to a selective deletion of alloreactive T cells by apoptosis. The regulation of the apoptosis pathway in allo-reactive T cells may be critical for the establishment of tolerance and merits further investigations as a potential tolerance-promoting pharmacological target.

Apoptosis

The elimination of superfluous, damaged or dangerous cells is pivotal for the development and the survival of multicellular organisms (Vaux and Korsmeyer 1999). For this purpose, animals developed a controlled, genetically programmed form of cell death called **apoptosis** (from the ancient Greek, "leaves falling from a tree"). Apoptosis has been evolutionarily conserved in metazoans and is characterized by **morphological features** that distinguish it from other forms of cell death. In contrast to the cell and organelle swelling and the early disruption of the cell membrane typically seen in necrosis, apoptotic cells present nuclear chromatin condensation, followed by cytoplasmatic budding and cell shrinkage. Finally, cell fragments called apoptotic bodies are produced and quickly removed by phagocytic cells without inducing an inflammatory response or any damage to the surrounding tissue (Kumar, Cotran et al. 2003; Green, Ferguson et al. 2009). The whole process is 20 times faster than mitosis and even substantial apoptosis may be histologically inapparent (Melino 2001). Therefore, although morphological features of apoptosis have been described since the middle of the nineteenth century, the term apoptosis to describe a common type of cell death was only introduced in 1972 (Kerr, Wyllie et al. 1972), but it was not generally credited until the 1980s when the group of Robert Horvitz first defined the genetics of apoptosis in *Caenorhabditis elegans* (Horvitz, Sternberg et al. 1983; Horvitz, Shaham et al. 1994).

Apoptosis is involved in **embryonic development**, playing an important role in morphogenesis, removing vestigial or sexual incongruent tissues and generating lacunae and separation of tissue layers (Yin and Dong 2009). In **adult animals**, the balance between mitosis and apoptosis controls the number of cells in different tissues, a particularly important issue in highly proliferating tissues (such as in the intestinal epithelium, the endometrium or in the hematopoietic system). Furthermore, apoptosis is a fundamental effector and regulatory mechanism of the immune system and has a crucial role in the elimination of cells with an irreparable DNA damage. As a result, apoptosis is inevitably involved in the pathogenesis of a variety of **diseases**: defects in the control of apoptosis notoriously result in cancer and autoimmunity, but there is growing evidence that apoptosis dysregulation is also critical for ischemic, neurodegenerative and infectious disorders (Hotchkiss, Strasser et al. 2009).

The control of life and death in each single cell of a multicellular organism is the result of the integration of a plurality of signals generated in the cell itself or provided by the environment. The molecular regulation of apoptosis in mammalian cells reflects this dualistic concept (Fig. 7): two converging signaling pathways culminate in the activation of **caspases**, a family of aspartate-specific cystein proteases. Caspases are synthetized in an inactive form (zymogen) and can be activated through three different mechanisms. (1) Caspases can activate caspase zymogens resulting in a "caspase cascade" that enormously amplify the pro-apoptotic signal. (2) The recruitment of procaspases in protein complexes that lead to a clustering of the zymogens leads to their self-processing (induced proximity). (3) An association with other regulatory proteins may be required for the full activation of the enzymatic activity (described for caspase 9 and Apaf-1). Activation of the **caspase cascade** leads to the execution of the apoptosis process. Caspases cleave essential cellular proteins (such as nuclear lamins and components of the cytoskeleton), activate the caspase-activated DNase responsible for the generation of the typical DNA ladder and other substrates, resulting in cell death and

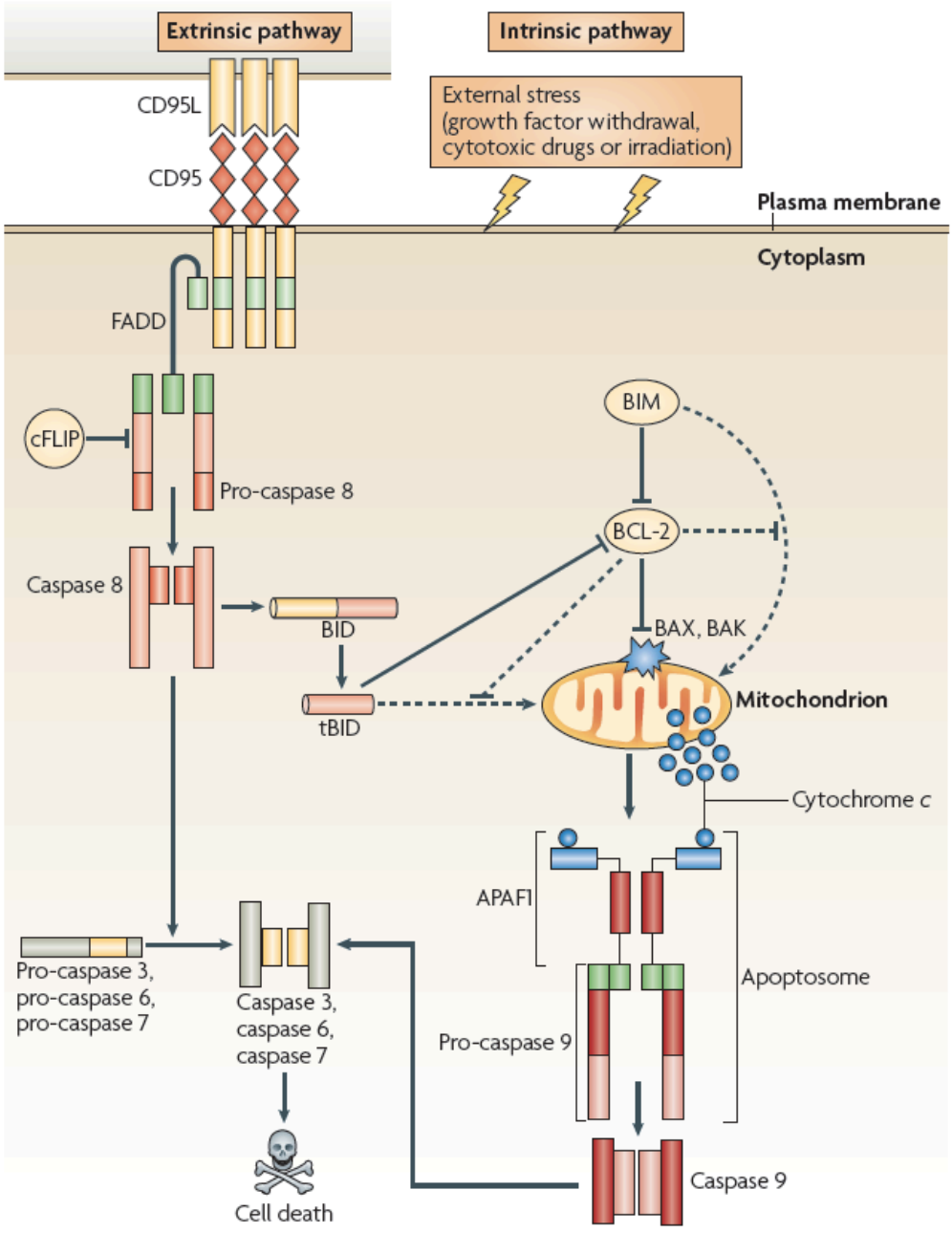

Fig. 7. A simplified scheme of the apoptosis pathway (Bouillet and O'Reilly 2009)

in the morphological changes typically observed in apoptotic cells (Hengartner 2000).
The **extrinsic apoptotic pathway** is triggered by cell-surface receptors of the tumor necrosis factor (TNF) receptor superfamily, called death receptors (DRs), and characterized by the presence of a cytoplasmatic sequence known as death domain, which enables the initiation of the death signal (Nagata 1997). Currently, six DRs have been characterized: **Fas** (CD95, APO-1), TNF-R1, TRAIL-R1, TRAIL-R2, DR3 and DR6. Binding of the natural ligand leads to oligomerization of the receptor and to the formation of large protein complexes at the cell membrane, known as **DISC** (death-inducing signaling complex). Formation of DISC leads to the accumulation of the pro-caspase 8 (and pro-caspase 10 in humans) in close proximity resulting in their activation by induced

Anti-apoptotic	Bcl-2	Lymphocytes Melanocytes Kidney	
	Bcl-xL	Lymphocytes Megakaryocytes Platelets	Embryonic lethality
	Bcl-w		Male sterility
	Mcl-1	HSCs Myeloid cells	
	A1/Bfl-1	Lymphocytes Granulocytes Mast cells	
	Bcl-B	?	
Pro-apoptotic (BH3-only)	Bad	Pancreas	
	Bid	Hepatocytes Myeloid cells	Link extrinsic-intrinsic pathway
	Bik		Spermatogenesis
	Bim	Lymphocytes	
	Bmf	Lymphocytes	
	Noxa	Lymphocytes	Irradiation
	PUMA	Lymphocytes	DNA damage
	Hrk/DP5	?	
Pro-apoptotic (Multi-domain)	Bax		Mitocondrial membrane permeabilization
	Bak		
	Bok	?	

Tab. 4. Mammalian Bcl-2 family proteins, their expression and function (Yin and Dong 2009, modified)

proximity. As initiators of the extrinsic apoptosis pathway, caspase 8 subsequently activates the executioner caspases 3, 6 and 7 (Krammer, Arnold et al. 2007).

The **intrinsic apoptotic pathway** (or mitochondrial pathway) is induced by the permeabilization of the outer mitochondrial membrane followed by the release of cytochrome c and other apoptogenic proteins such as SMAC into the cytoplasm. Cytochrome c leads to the formation of a large protein complex (called **apoptosome**) with Apaf-1 (apoptotic protease activating factor 1) and pro-caspase-9, the initiator caspase of the intrinsic pathway (Li, Nijhawan et al. 1997). The **Bcl-2 family** (B cell lymphoma 2) is an evolutionarily conserved family of proteins that control the permeabilization of

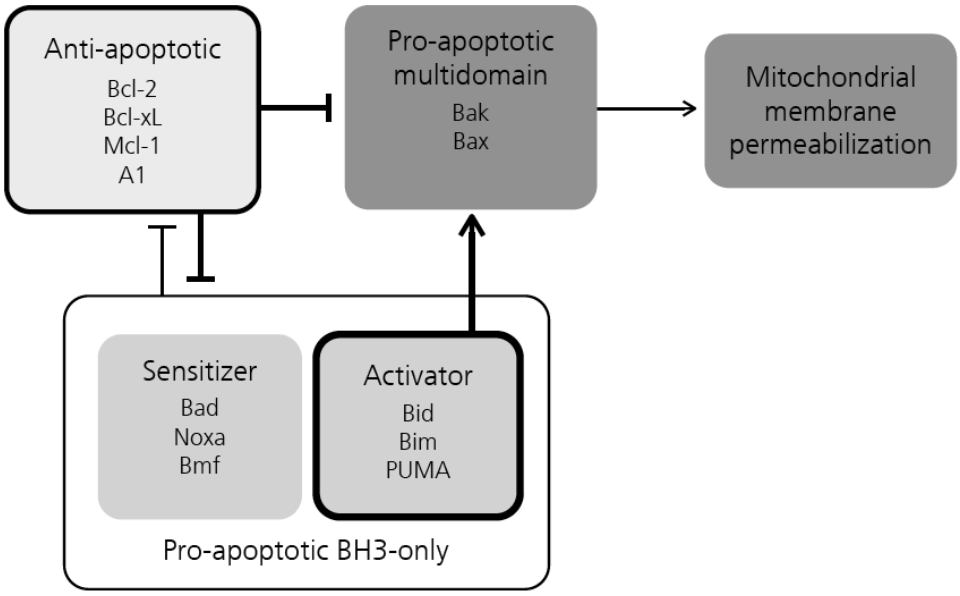

Fig. 8. Molecular interactions of Bcl-2 proteins

mitochondrial membrane and is therefore a pivotal regulator of the intrinsic pathway (Hengartner and Horvitz 1994; Wei, Zong et al. 2001). Bcl-2 family members share sequence homology in four domains (BH1-4) and can be divided in three groups according to their function and structure (Tab. 4). Anti-apoptotic Bcl-2 proteins contain all BH domains; among the pro-apoptotic factors a group contains BH1, BH2 and BH3 (multidomain), whereas another group is characterized by the only presence of the BH3 domain (BH3-only proteins) (Strasser 2005). Several models have been proposed to explain the molecular interactions between Bcl-2 proteins. **Bax** and **Bak** are directly involved in the permeabilization of the outer mitochondrial membrane and deletion of both of them (but not one of them) renders the cell completely resistant to the activation of the intrinsic apoptosis pathway (Wei, Zong et al. 2001). Anti-apoptotic factors inhibit Bax and Bak and mutually interact with **BH3-only** proteins. A subgroup of BH3-only proteins (including Bid, Bim and PUMA), called **activators**, has been shown to directly activate Bax and Bak, whereas **sensitizers** (such as Bad, Noxa and Bmf) exert their pro-apoptotic effect through the inactivation of anti-apoptotic factors (Kuwana, Bouchier-Hayes et al. 2005; Letai 2008). This results in a complex multi-factorial model that represents the basis for a dynamic regulatory mechanism (Fig. 8) (Strasser, Puthalakath et al. 2008).

The activity of the different members of the Bcl-2 family is finely regulated at many levels in response to disparate stimuli. For example, DNA damage induces the transcription of PUMA, Noxa, Bid in a p53-dependent manner (Oda, Ohki et al. 2000) and UV irradiation induces the translocation of Bim from the cytoskeleton to the mitochondrial membrane (Puthalakath, Huang et al. 1999). A particular function is provided by the BH3-only factor **Bid**, which connects the two apoptosis pathways (Fig. 7). Bid is activated by caspase 8 and induces an amplification of the extrinsic pathway via an indirect activation in the instrinsic pathway (Chou, Li et al. 1999).

Additional levels of **complexity** are provided by regulators of the extrinsic apoptosis pathway (such as c-FLIP) (Thome and Tschopp 2001), by the role of caspases and Bcl-2 proteins in cellular processes not directly related to apoptosis (Kennedy, Kataoka et al. 1999), by inhibitors of caspases (inhibitors of apoptosis, IAPs) (Jost, Grabow et al. 2009; Kaufmann, Strasser et al. 2012)

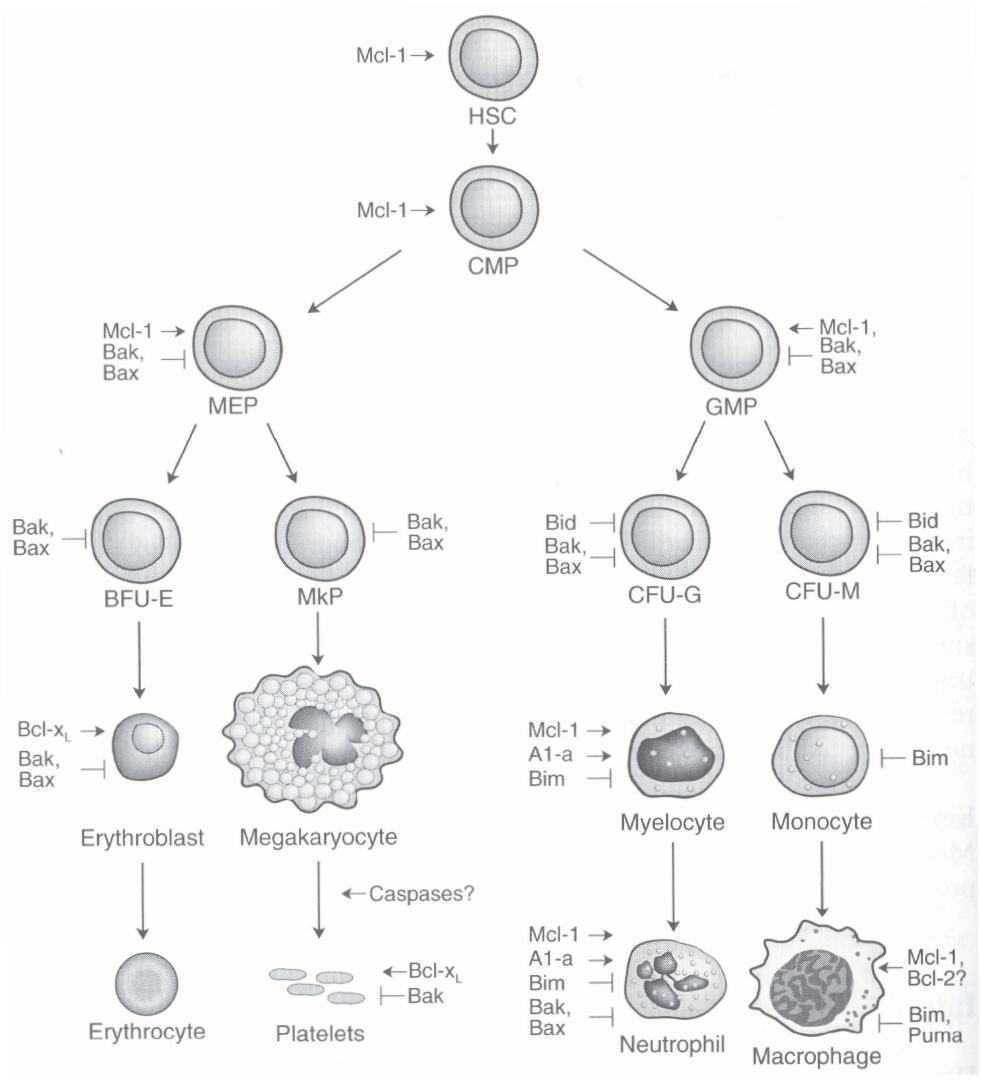

Fig. 9. The role of Bcl-2 proteins in hematopoiesis (Yin and Dong 2009)
HSC: hematopoietic stem cell, CMP: common myeloid progenitor, MEP: megakaryocyte/erythroid progenitor, GMP: granulocyte/macrophage progenitor, BFU-E: burst-forming unit-erythroid, MkP: megakaryocyte progenitor, CFU-G: colony-forming unit granulocyte, CFU-M: colony-forming unit macrophage.

and by the role of other organelles (such as the endoplasmatic reticulum) in the modulation of apoptosis. Thus, the regulation of apoptosis is tightly interconnected with the function of the cell and promptly responds to stimuli from the environment. "Such social control of life and death are vital in complex multicellular networks such as the immune system and the nervous system, where communication between cells is crucial" (Melino 2001).

Role of apoptosis in the immune system

The regulation of cell survival is essential for the correct functioning of the immune system: it plays a pivotal role in the generation of immune cells, it exerts important effector and regulatory functions and it is required for the maintenance of immune homeostasis.

Apoptosis – and particularly the Bcl-2 family - is actively involved in **hematopoiesis**, the process by which HSCs generate mature blood and lymphatic cells through multiple rounds of division and differentiation. The survival of mature blood and lymphatic cells is genetically pre-determined during development, and all cells are intrinsically **programmed to die** in the absence of external stimuli. For this purpose, Bcl-2 factors are specifically regulated in different cell lineages during hematopoiesis resulting in a di-

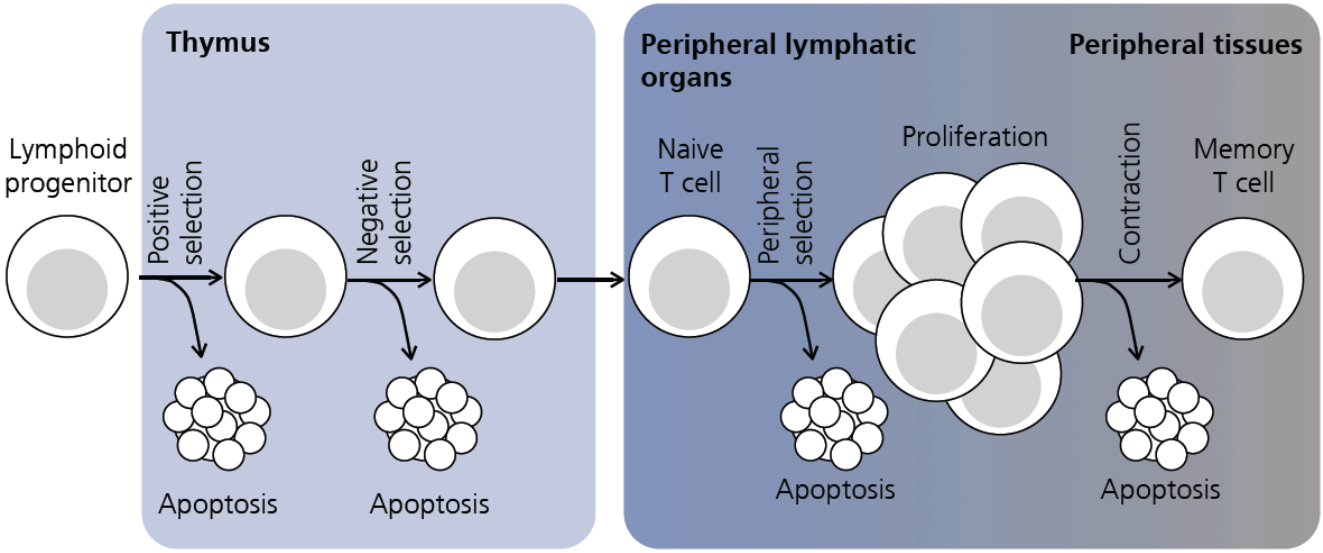

Fig. 10. Critical role of apoptosis in T cell homeostasis

verse expression of pro- and anti-apoptotic factors among the different cell populations (Fig. 9) (Yin and Dong 2009). The survival of HSCs critically depends on the expression of Mcl-1 (Opferman, Iwasaki et al. 2005) and the same factor inhibits the pro-apoptotic effect of Bim in neutrophils (Leuenroth, Grutkoski et al. 2000), whereas Bcl-xL counteracts the pro-apoptotic effect of Bak in platelets (Josefsson, James et al. 2011).

The regulation of the apoptosis pathway in **lymphocytes** is much more complex. Here we will focus on T lymphocytes (Starr, Jameson et al. 2003) (Fig. 10). **T cells** originate from common lymphoid progenitors that migrate from the bone marrow into the thymus, where they proliferate extensively and start to differentiate (Murphy, Travers et al. 2008). In the first stages of development these cells lack most of the surface molecules and do not express a T cell receptor (TCR). Their survival depends on the presence of IL-7, which induces the expression of Bcl-2 and Mcl-1 to counteract the pro-apoptotic effect of Bim (Pellegrini, Bouillet et al. 2004). In the next development steps, thymocytes undergo TCR gene rearrangement, first at the β-chain locus, then at the α-chain locus. Failure to resolve double-strand DNA breaks during the rearrangement process leads to p53-dependent apoptosis (Jiang, Lenardo et al. 1996). The expression of a functional pre-T and T cell receptor is required for thymocytes to survive from the double negative (CD4-CD8-) to the double positive (CD4+CD8+) and finally to single positive CD4 or CD8 stage. This last step, called **positive selection**, is mediated by the up-regulation of A1 (Mandal, Borowski et al. 2005) and ensures that only cells expressing a functional TCR that can bind to an MHC/peptide complex expressed by thymic epithelial cells will survive. As discussed in chaper 1, **negative selection** is the key checkpoint for central tolerance. Thymocytes with a high affinity to MHC/self-peptide complexes are deleted from the repertoire by a Bim-dependent activation of the intrinsic apoptosis pathway (Bouillet, Purton et al. 2002). As a result, approximately 95% of developing thymocytes die by activation of the intrinsic apoptosis pathway during the different stages of differentiation.

The CD4 or CD8 single positive T cell that survive this process migrate to the peripheral lymphatic organs as **naïve T cells** until they encounter an antigen. The survival of naïve T cells depends on the inhibition of the intrinsic apoptosis pathway by Bcl-2, which counteracts the pro-apoptotic effect of Bim (Wojciechowski, Tripathi et al. 2007). The expression of Bcl-2 depends on IL-7 and requires a basal activity of calcineurin (Tan,

Dudl et al. 2001; Manicassamy, Gupta et al. 2008). Interestingly, CD8 T cells are more sensitive to the loss of prosurvival signaling than CD4 T cells. The regulation of apoptosis dramatically changes after antigen recognition: **T cell activation** triggers the expression of proteins with anti-apoptotic function such as c-FLIP, Bcl-2, Bcl-xL, A1, XIAP and with pro-apoptotic function such as Fas, TRAIL-R1, Bim and Nur77 (Sandalova, Wei et al. 2004; Brenner, Krammer et al. 2008). The precise regulation of the intrinsic and the extrinsic apoptosis pathway is under the control of costimulatory molecules and cytokines, and is required to promote the survival of reactive and proliferating cells during the immune response, but also to ensure elimination of the majority of them after antigen clearance (Rathmell and Thompson 2002). **T cell contraction** is the result of cooperation between the extrinsic and the intrinsic apoptosis pathway (Weant, Michalek et al. 2008). In parallel, a small percentage of reactive T cells survive as **memory cells**. A down-regulation of Bim via regulation of its proteosomal degradation seems to be crucial for the generation of memory cells (Sabbagh, Srokowski et al. 2006; Sabbagh, Pulle et al. 2008), and the balance between Bcl-2, Bcl-xL and Bim is critical for their long-term survival (Wojciechowski, Tripathi et al. 2007).

Apart from its role in the development and in the maintenance of immune homeostasis, apoptosis is an important **effector function** of **cytotoxic cells** (principally CD8 T cells and natural killer cells). To eliminate intracellular pathogens or potentially dangerous cells, cytotoxic lymphocytes induce cell death by activation of the extrinsic apoptosis pathway (mostly triggering Fas), or releasing the content of cytotoxic granules. Although the cytotoxic granzymes and perforins induce cell death by creating pores in the plasma membrane, the process can be amplified by activation of the apoptosis pathway, for example by the cleavage of Bid by granzyme B (Cullen and Martin 2008). Similar mechanisms are also important in the complex intercellular **regulation** of the immune system. Regulatory T cells regulate the survival of effector CD4 and CD8 T cells by inducing apoptosis through TRAIL or indirectly by a deprivation of cytokines (Ren, Ye et al. 2007). Moreover, Th1 differentiated CD4 T cells can kill chronically infected macrophages by activation of the Fas signaling to release bacteria to be destroyed by fresh macrophages (Murphy, Travers et al. 2008).

Because of the critical role of apoptosis in the maintenance of immune homeostasis and regulation, a dysregulation of apoptosis pathways is a common cause of **autoimmunity** and **lymphoproliferative disorders** (Rieux-Laucat, Le Deist et al. 2003). Studies in transgenic animals have been crucial for the characterization of these processes: ***lpr*** mice (mutation in Fas) and mice over-expressing Bcl-2 develop a lupus-like autoimmune disease (Andrews, Eisenberg et al. 1978), and Bim knock-out mice present splenomegaly, a progressive accumulation of lymphocytes and are prone to autoimmunity (Bouillet, Metcalf et al. 1999). On the other hand, Bcl-2 knock-out mice are characterized by lymphopenia (Veis, Sorenson et al. 1993). Similar disorders have been described in humans: mutations in Fas or FasL result in the autoimmune lymphoproliferative syndrome (**ALPS**), characterized by lupus-like autoimmunity, hypergammaglobulinemia, B-cell lymphocytosis and predisposition to develop lymphomas (Bidere, Su et al. 2006). An additional link between apoptosis and autoimmunity may result from the mechanism involved in the **clearance of apoptotic cells**. One of the most important characteristics of apoptosis consists in the absence of inflammation after phagocytosis of apoptotic bodies, and it is known that failure or delay in removing dying cells can induce autoimmunity. The mechanism determining the tolergenic properties of apoptotic cells is uncompletely understood (Green, Ferguson et al. 2009).

Pharmacological apoptosis modulation

The apoptosis pathway is a promising pharmacological target with various potential clinical applications (Hotchkiss, Strasser et al. 2009). In general, diseases with an excessive

cell death, such as myocardial or cerebral ischemia, cisplatin nephrotoxicity or neurodegenerative disorders might be beneficially influenced by an **inhibition of apoptosis** (Letai 2005). Currently, the most significant clinical application of this approach is represented by the administration of cyclosporine A in patients with acute myocardial infarction to reduce the infarction size by a modulation of the mitochondrial permeability-transition pores (Piot, Croisille et al. 2008), but several more selective strategies are being investigated in different experimental models (Kostic, Jackson-Lewis et al. 1997; Becattini, Sareth et al. 2004; Hamar, Song et al. 2004).

Apoptosis induction may be favorable to treat diseases characterized by a deficient cell death machinery. Apart from a potential clinical relevance for the rare immunological diseases caused by a primary defect in apoptosis regulation mentioned before, pro-apoptotic drugs may be indicated to treat cancer (Labi, Grespi et al. 2008). Dysregulation of apoptosis is a hallmark of **cancer** and neoplastic cells develop various strategies to evade apoptosis: for example, overexpression of Bcl-2 is present in approximately 85% of human follicular lymphoma (Tsujimoto, Finger et al. 1984). These mechanisms are not only involved in tumor pathogenesis, they also provide resistance to conventional cancer therapy (chemotherapy and radiotherapy), since these therapies also exert their anti-tumor effect by inducing apoptosis in cancer cells (Yin and Dong 2009). However, pro-apoptotic drugs should be used with caution. As a fundamental cellular function, apoptosis is universally functional in animal cells, and an uncontrolled activation of apoptosis would inevitably results in major **side effects**. This can partially be avoided by the development of drugs selectively targeting single molecular players in the apoptosis pathway. Several partially **redundant** mechanisms protect the cell from an undesired suicide (Bortner and Cidlowski 2002), and deletion of a single anti-apoptotic gene is mostly not lethal in transgenic mice (Veis, Sorenson et al. 1993; Hamasaki, Sendo et al. 1998; Harlin, Reffey et al. 2001). Therefore, the selective pharmacological modulation of single apoptotic factors is unlikely to induce generalized apoptosis, and because different cell types protect themselves from apoptosis using different mechanisms, a **selective deletion** of defined cell populations is theoretically possible using selective pro-apoptotic drugs. Finally, pro-apoptotic drugs can be used to interfere with **apoptosis-dependent processes**, such as the central and peripheral selection of lymphocytes.

Most efforts to develop drugs that trigger apoptosis have been made in the field of **oncology**. Stimulation of death receptors is an attractive and technically feasible approach (Ashkenazi 2002). However, the systemic application of Fas-L and TNFα resulted in major toxicities (Ogasawara, Watanabe-Fukunaga et al. 1993). In contrast, **TRAIL** represents a promising candidate, and human monoclonal antibodies to stimulate TRAIL-R1 or TRAIL-R2 are currently evaluated in clinical trials (Tolcher, Mita et al. 2007). An additional target of interest is the family of inhibitor of apoptosis proteins (IAPs): particularly, inhibitors of the X-linked inhibitor of apoptosis (**XIAP**) are in early clinical evaluation (Lacasse, Kandimalla et al. 2005). The most promising approach is the manipulation of the **Bcl-2 family** (Vogler, Dinsdale et al. 2009). Initial attempts to inhibit the anti-apoptotic function of Bcl-2 using unmodified BH3 domain peptides failed, because of the problem of intracellular de-

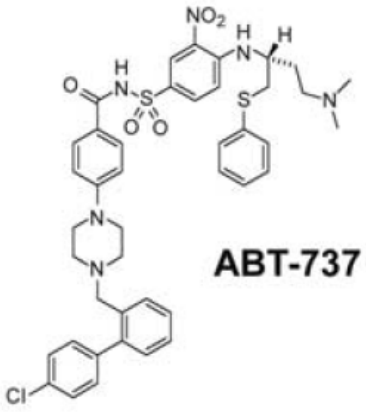

Fig. 11. Chemical structure of ABT-737 (Oltersdorf, Elmore et al. 2005)

livery. However, small molecule Bcl-2 inhibitors were identified by screening of chemical libraries or developed by iterative modulation of chemical structure based on NMR (Letai 2005). As a result, a series of Bcl-2 inhibitors are currently being tested in preclinical tumor models. Importantly, different compounds displayed a different binding affinity to the different anti-apoptotic members of the Bcl-2 family, which eventually determined their selectivity profile (Zhai, Jin et al. 2006). Three drugs are currently in advanced clinical evaluation (Khaw, Huang et al. 2011).

ABT-737 and **navitoclax** (ABT-263, Abbott, Fig. 11) bind with high affinity to Bcl-2, Bcl-xL and Bcl-w, but not to Mcl-1 and A1 (Oltersdorf, Elmore et al. 2005; Tse, Shoemaker et al. 2008). As a result, ABT-737 triggered apoptosis in tumor cells and sensitized them to chemotherapy, but tumors expressing Mcl-1 and A1 were resistant to the treatment (Yecies, Carlson et al. 2010). ABT-737 and navitoclax have the same Bcl-2 selectivity profile and displayed the same pharmacodynamic properties. However, navitoclax is characterized by a better solubility and bioavailability and was therefore used for clinical studies, whereas ABT-737 was further used in the experimental setting. Navitoclax has been tested in Phase 1 trials in patients with advanced lymphomas, small cell lung cancer and chronic lymphocytic leukemia (CLL) (tumors with high-level expression of Bcl-2) (Wilson, O'Connor et al. 2010; Gandhi, Camidge et al. 2011; Roberts, Seymour et al. 2011). In the CLL trial 9 of 26 patients achieved a partial response, but the results were less encouraging in small cell lung cancer. Patients displayed a moderate depletion of CD3 T cells within 14 days without an increased risk for infections, and a dose-dependent thrombocytopenia without major bleeding episodes. ABT-737-induced **thrombocytopenia** depends on the inhibition of Bcl-xL and is primarily due to a direct depletion of circulating platelets by apoptosis, although Bcl-xL inhibition can also induce a dysfunction in the generation of platelets in the bone marrow (Josefsson, James et al. 2011). Platelet survival is determined by the balance between Bcl-xL and Bak. Since platelets are anucleate cells, they receive their stock of both proteins during their generation in the bone marrow. Inhibiting Bcl-xL inevitably leads to an irreversible shift towards the pro-apoptotic side (Mason, Carpinelli et al. 2007). Phase 2 trials with navitoclax as a single agent and in combination with conventional chemotherapy are in progress.

Clinical studies using the pan-Bcl-2 inhibitor **obatoclax** resulted in major neuropsychiatric side effects and in a lack of significant single agent activity (Hwang, Kuruvilla et al. 2010). The Bcl-2 / Bcl-xL / Mcl-1 inhibitor **AT-101** displayed a limited clinical activity as single agent in patients with CLL, prostate cancer and small cell lung cancer, and induced a high incidence of severe gastrointestinal side effects (James, Castro et al. 2006). Interestingly, in contrast to navitoclax, neither obatoclax nor AT-101 induced the expected Bcl-xL-inhibition dependent thrombocytopenia, suggesting that their lower binding affinity may not be sufficient to achieve a clinically relevant response (Khaw, Huang et al. 2011).

Previous studies tried to inhibit undesired **immune reactions** targeting the apoptosis pathway. In the 1990s it was recognized that up-regulation of **Fas** occurs in T cells after antigen recognition, opening the opportunity for a selective depletion of activated T cells by Fas-L to prevent allograft rejection (Owen-Schaub, Yonehara et al. 1992). Apart from the limitation of this approach because of toxic side effects (Ogasawara, Watanabe-Fukunaga et al. 1993), the hypothesized immunosuppressive effect could not be achieved, because of an inactivation of the extrinsic apoptosis pathway in early-activated lymphocytes (O'Flaherty, Ali et al. 1998). In contrast, an immunosuppressive effect of **Bcl-2 inhibitors** has recently been demonstrated in mouse models of autoimmunity (Bardwell, Gu et al. 2009). The immuno-modulatory mechanism of this approach and its potential to control allograft rejection and induce tolerance have been investigated in this project.

Chapter 3: The BH3-mimetic ABT-737 inhibits allogeneic immune responses

Pietro E. Cippà[1,2], Anna K. Kraus[1,2], Ilka Edenhofer[1,3], Stephan Segerer[2,3], Jin Chen[1,2], Martin Hausmann[4], Yang Liu[1,3], Annick Guimezanes[5], Philip D. Bardwell[6], Rudolf P. Wüthrich[1,2], Thomas Fehr[1,2]

[1] Institute of Physiology, University of Zürich, Switzerland
[2] Division of Nephrology, University Hospital Zürich, Switzerland
[3] Institute of Anatomy, University of Zürich, Switzerland
[4] Division of Gastroenterology and Hepatology, University Hospital Zürich, Switzerland
[5] Centre d'Immunologie de Marseille-Luminy, Université de la Méditerranée, Marseille, France
[6] Abbott Bioresearch Center, Worcester, USA

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Abstract
Apoptosis controls the adaptive immune system through regulation of central and peripheral lymphocyte deletion. Therefore, substances that selectively interact with the intrinsic apoptosis pathway in lymphocytes offer unexplored opportunities to pharmacologically modulate the immune response. Here we present evidence that the BH3-mimetic ABT-737 suppresses allogeneic immune responses. *In vitro*, ABT-737 prevented allogeneic T cell activation, proliferation and cytotoxicity by apoptosis induction, but without impairing the physiological functions of remaining viable T cells. *In vivo*, ABT-737 was highly selective for lymphoid cells and inhibited allogeneic T and B cell responses after skin transplantation. The immunosuppressive effect of ABT-737 was markedly increased in combination with low-dose cyclosporine A, as shown by the induction of long-term skin graft survival without significant inflammatory infiltrates in 50% of the recipients in a MHC class I single antigen mismatched model. Thus, pharmacological targeting of Bcl-2 proteins represents a novel immunosuppressive approach to prevent rejection of solid organ allografts.

Introduction

The identification of alternative pharmacological targets to suppress allo-specific immune responses is a fundamental step in the development of new drugs aiming at the optimization of long-term outcome after solid organ transplantation (Halloran 2004). It has been shown that the modulation of apoptosis in lymphocytes is responsible for central and peripheral repertoire selection (Bouillet, Purton et al. 2002; Rathmell and Thompson 2002) and controls the deletion of alloreactive lymphocytes in the induction of peripheral transplantation tolerance (Wells, Li et al. 1999). Therefore, targeting apoptosis pathways in lymphocytes represents a potential novel strategy for immunosuppression.

Two distinct but interconnected pathways regulate apoptosis in mammalian cells: the extrinsic pathway is mediated by death receptors at the cell surface (e.g. by FAS or TNF-receptor), whereas the intrinsic (or mitochondrial) pathway is under the control of

pro- and anti-apoptotic members of the Bcl-2 family (Hotchkiss, Strasser et al. 2009). Selective small-molecule Bcl-2 antagonists have rationally been developed for the treatment of tumors (Oltersdorf, Elmore et al. 2005). The Bcl-2 inhibitor ABT-737 and its orally bioavailable counterpart ABT-263 bind the anti-apoptotic proteins Bcl-2, Bcl-XL and Bcl-w (but not A1 and Mcl-1) and activate the intrinsic apoptosis cascade in human and murine tumor cells (Hann, Daniel et al. 2008). While research on Bcl-2 inhibitors focused on cancer biology, this novel class of drugs offers the opportunity for selective interaction with a fundamental pathway in mammalian cells and could find various clinical applications outside of oncological therapy. Interestingly, it has been shown that ABT-737 has a favorable effect on various autoimmune diseases in mice and inhibits B and T cell immune responses (Bardwell, Gu et al. 2009; Carrington, Vikstrom et al. 2010). Moreover, Carrington *et al.* recently described a beneficial impact of ABT-737 on glucose levels after islet transplantation from (C57BL/6 x SJL)F_1 to spontaneous non-immune diabetic RIP-H-2K^b mice (Carrington, Vikstrom et al. 2010). However, BH3-mimetics have never been tested in stringent models of allograft rejection.

Here we present evidence that the BH3-mimetic ABT-737 suppresses allogeneic T cell responses *in vitro* due to a clone size reduction in the alloreactive T cell population by apoptosis induction. *In vivo*, ABT-737 selectively induced apoptosis in lymphatic tissues and potently inhibited allogeneic T and B cell responses after skin transplantation in mice synergistically with cyclosporine A (CsA).

Materials and Methods

Mice. C57BL/6 (B6, H-2^b), CBA (H-2^k), B6.C-H2Kbm1 (bm1, H-2^{bm1}), BALB/c (H-2^d) and BM3.3 (H-2^k) mice were housed in specific pathogen-free conditions at the University of Zürich. Bm1 mice (7 nucleotides mutation in the H-2K^b locus) were purchased from The Jackson Laboratory (Bar Harbor, USA) (Schulze, Pease et al. 1983). BM3.3 mice express on all CD8 T cells a transgenic T cell receptor (TCR) selective for a naturally processed octapeptide bound to the allogeneic MHC class I molecule H-2K^b (Auphan, Curnow et al. 1994; Guimezanes, Barrett-Wilt et al. 2001). All animal experiments were performed according to protocols approved by the legal authority (Veterinary Office of the Canton of Zürich).

In vivo experiments. Full thickness tail skin (about 0.5-1.0 cm^2) from donor mice was grafted. Recipient mice were treated from day 5 before grafting until allograft rejection. Skin grafts were considered rejected when <10% of the graft remained viable. In some experiments recipient mice were sacrificed at day 8 after transplantation and the transplanted tissue was harvested for histological evaluation. ABT-737 was provided by Abbott Bioresearch (Worcester, USA) and was injected intra-peritoneally (i.p.) at 50 mg/kg/day. Vehicle consisted of polyethylene glycol, Tween 80, dextrose solution and DMSO. CsA was purchased from Sigma-Aldrich (Buchs, CH) and injected subcutaneously at 10 mg/kg/day.

Mixed lymphocyte reaction (MLR) and cell-mediated lympholysis (CML) assay. MLR were performed with BM3.3 splenocytes stimulated by irradiated CD8 T cell-depleted splenocytes from B6 (allogeneic) and CBA or BM3.3 (syngeneic) mice. Splenocytes were sorted by magnetic cell separation according to the protocols of Miltenyi Biotec (Bergisch Gladbach, D) to allow a selective analysis of alloantigen-specific CD8 T cells. ABT-737 or DMSO-containing vehicle were added to the MLR at the beginning of the culture. For the CML assay B6 splenocytes were cultured with irradiated BALB/c (allogeneic) and B6 (syngeneic) lymphocytes during 5 days (stimulation phase). Thereafter, ^{51}Cr-labelled concanavalin-A-stimulated BALB/c lymphocytes were added to the serially diluted culture for 4 hours (killing phase), and alloantigen-specific cytotoxicity was assessed by measurement of ^{51}Cr release in the supernatant. For a compensation of the alloreactive clone size reduction in certain experiments the same number of viable ABT-737 treated responder cells was used for the kill-

ing phase as measured in vehicle control cultures. Alloantigen-specific lysis was calculated as: % specific lysis = (experimental release – spontaneous release) / (total release – spontaneous release)*100.

Flow cytometry (FACS) and detection of allospecific antibodies. FACS was performed with a BD-FACSCanto (Becton Dickinson, Allschwil, CH). Anti-mouse CD3-FITC, CD4-PE, CD8-APC, CD8-PE, CD45R/B220 PE-Cy5, anti-IgG-FITC, anti-IgM-PE, annexin V-FITC and propidium iodide (PI) were purchased from eBioscience (Frankfurt, D), anti-mouse CD25-PE/Cy7 and interferon-γ (IFN-γ)-APC from Biolegend (Uithoorn, NL), carboxyfluorescein succinimidyl ester (CFSE) from Promega (Dübendorf, CH). Allospecific antibodies were detected by FACS: after Fc-receptor blockade, allogeneic splenocytes were incubated with recipients' serum and subsequently stained with a secondary anti-mouse anti-IgG and anti IgM antibody.

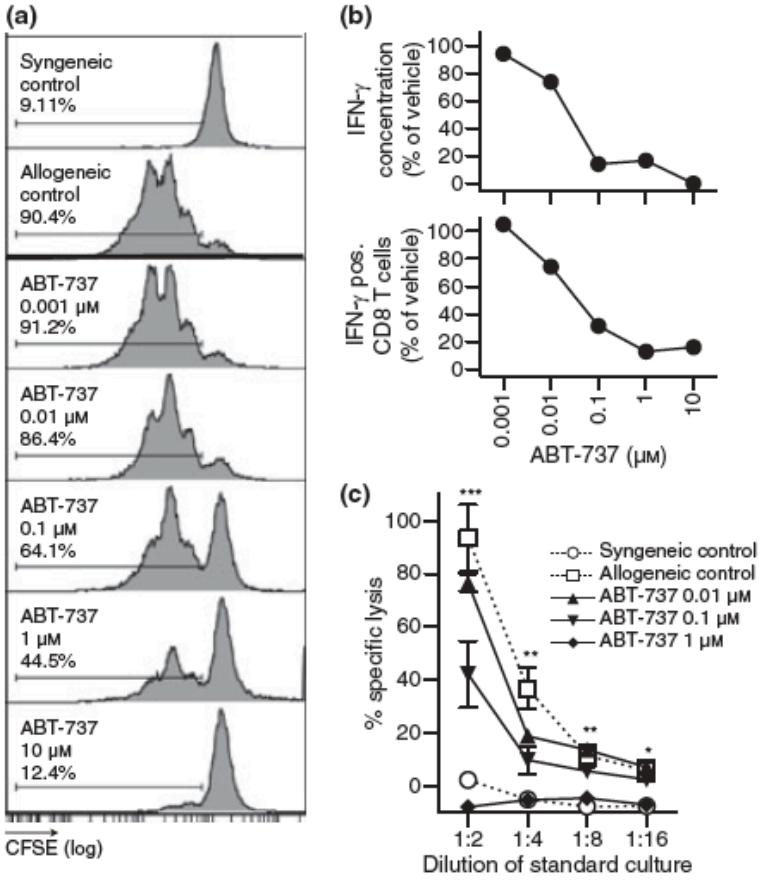

Fig. 12. ABT-737 suppresses allogeneic T cell responses *in vitro.*

A) MLR with transgenic BM3.3 splenocytes stimulated with CD8-depleted irradiated B6 splenocytes. After 3 days of MLR, ABT-737 suppressed CD8 T cell proliferation in a concentration-dependent manner as shown by CFSE-dilution in FACS. Representative results of one of three independent experiments are shown.

B) ABT-737 inhibited IFN-γ synthesis, as shown by reduction of IFN-γ concentration in the culture supernatant measured by ELISA (3 independent experiments). The effect on allo-reactive BM3.3 CD8 T cells was confirmed determining the number of IFN-γ positive transgenic CD8 T cells in an additional independent experiment. Percent of values measured in cultures exposed to vehicle are given.

C) B6 lymphocytes were stimulated with BALB/c splenocytes in the presence of ABT-737 or DMSO-containing vehicle during 5 days. Allo-antigen specific cytotoxicity was tested in a ^{51}Cr release assay against concavalin A-stimulated BALB/c lymphocytes. ABT-737 exposure during the stimulation phase suppressed alloantigen-specific cytotoxicity in a concentration-dependent manner. Representative results of one of three independent experiments are shown (one-way ANOVA comparing the four allogeneic stimulated groups, * $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Mean fluorescence intensity (MFI) was determined in FACS gating on B220 negative cells.

ELISA, Western blotting and blood analyses. IFN-γ ELISA was performed using a kit purchased from Biolegend (Uithoorn, NL) and used according to the manufacturer's protocol. Western blot analysis was performed using a rabbit anti-caspase-3 antibody (Cell Signaling Technology, Danvers, USA). A peroxidase-conjugated donkey anti-rabbit IgG (GE Healthcare, Buckinghamshire, UK) was used as secondary reagent. Hematological analyses were performed in the laboratory of the Division of Hematology at the University Hospital Zürich with an ADVIA 2120 flow cytometer (Siemens, Eschborn, D).

Histology, immunohistochemistry, TUNEL and analysis of isolated intestinal epithelial cells. Histology and immunohistochemistry were performed as previously described (Segerer, Hudkins et al. 2002). Monoclonal anti-CD3 antibody was purchased from AbD Serotec (Düsseldorf, D), anti-CD8a from OriGene (Rockville, USA). For detection of apoptotic cells by immunohistochemistry the monoclonal antibody F7-26 (Chemicon, International, Inc. Temecula, USA) was used as previously described (Segerer, Eitner et al. 2002). F7-26 binds to single-stranded DNA after thermal denaturation. A peroxidase-conjugated monoclonal rat anti-mouse IgM antibody (Zymed, San Francisco, USA) was used as secondary reagent. Apoptosis in intestinal mucosa associated lymphatic tissue was quantified by TUNEL technology with the In Situ Cell Death Detection Kit (Roche, Basel, CH) as described by the manufacturer. Intestinal epithelial cells were isolated and analyzed by FACS as previously described (Grossmann, Walther et al. 2003).

Statistics. Student t-test, Mann-Whitney test, ANOVA-test or Fisher's exact test were used to compare values between groups as appropriate; skin graft survival was compared by log-rank test. Graph Pad Prism Software Version 5.0 was used for calculations.

Results

Bcl-2 inhibition suppresses allogeneic T cell responses in vitro.

The Bcl-2 inhibitor ABT-737 was first tested *in vitro* in a mixed lymphocyte reaction (MLR) model. To analyze the immunosuppressive effect of ABT-737 on a homogeneous population of alloreactive T cells we used the transgenic mouse model BM3.3. The BM3.3 mouse expresses on all CD8 T cells a transgenic TCR specific for a naturally processed octapeptide bound to the allogeneic MHC class I molecule H-2K^b (Auphan, Curnow et al. 1994; Guimezanes, Barrett-Wilt et al. 2001). After 3 days of a classical MLR culture with BM3.3 responders and irradiated B6 stimulators we registered a potent, concentration-dependent inhibition of transgenic CD8 T cell activation (data not shown), proliferation (CFSE dilution, Fig. 12A) and IFN-γ synthesis (ELISA of culture supernatant and intracellular IFN-γ staining in transgenic CD8 T cells, Fig. 12B) in cultures exposed to ABT-737. Analogous results were observed at the cytotoxicity level in a cell-mediated lympholysis (CML) assay using the BM3.3 transgenic model (data not shown), and in the non-transgenic fully MHC-mismatched combination B6 to BALB/c (Fig. 12C).

Mechanism of immunosuppression by Bcl-2 inhibition in vitro.

Previous studies have shown that ABT-737 initiates the intrinsic apoptosis cascade by mitochondrial release of cytochrome c (Oltersdorf, Elmore et al. 2005). However, apart from their role as apoptosis regulators, Bcl-2 proteins seem to be involved in additional cellular functions in lymphocytes (Shibasaki, Kondo et al. 1997; Ludwinski, Sun et al. 2009). Here we show that the immunosuppressive effect of ABT-737 results from clone size reduction in the alloreactive T cell population by apoptosis induction. However, the fraction of alloreactive CD8 T cells that remained viable after exposure to a concentration of ABT-737 that did not induce a complete T cell deletion were not impaired in their physiological effector func-

tions. For this purpose, we analyzed the fate of alloreactive lymphocytes in culture after treatment with ABT-737. Within hours ABT-737 induced apoptosis in resting lymphocytes as shown by phosphatidylserine exposure (annexin V positivity) in PI negative cells by FACS analysis (Fig. 13A) and by caspase 3 activation in Western blot (Fig. 13B). Similar results were obtained for wild type and for transgenic BM3.3 lymphocytes. The specific examination of transgenic alloreactive CD8 T cells in the BM3.3 to B6 model revealed that ABT-737 markedly increased the number of apoptotic cells in this population after 3

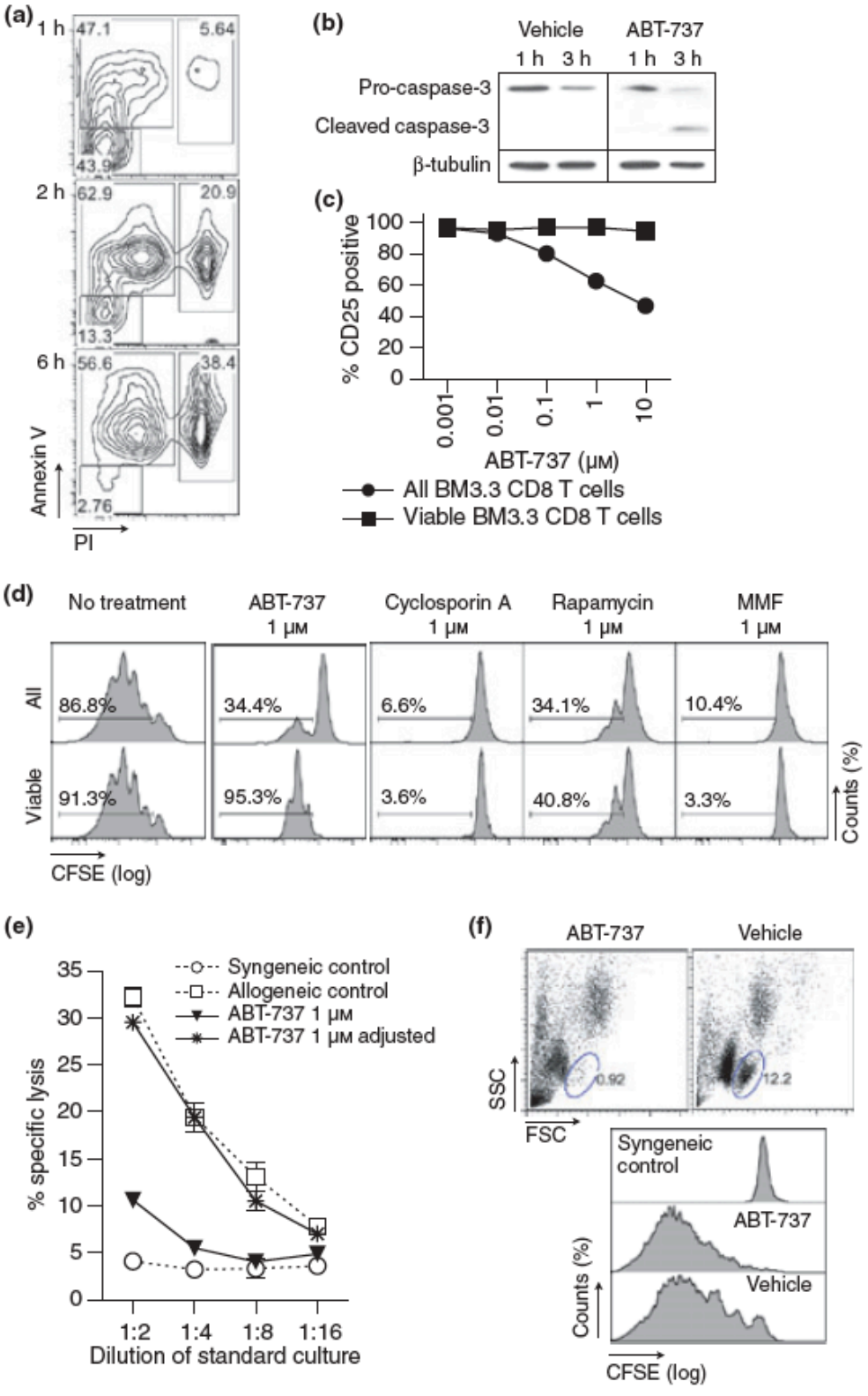

Fig. 13. The immunosuppressive mechanism of ABT-737.
A-B) ABT-737 (5 µM) induced apoptosis in B6 splenocytes within hours as shown by exposure of phosphatidylserine in propidium iodide negative cells (FACS) (A) and caspase 3 activation (Western blot) (B).
C-D) In MLR with BM3.3 splenocytes stimulated by irradiated CD8-depleted B6 splenocytes ABT-737 inhibited CD8 T cell activation (C) and proliferation (D) in a concentration-dependent manner if all responder BM3.3 TCR cells were considered. In contrast, activation and proliferation were not impaired by ABT-737 in a selective analysis of viable (PI negative) responder BM3.3 TCR. Similar results were obtained from several experiments with different MLR conditions. Representative data obtained after 3 days of MLR are shown and compared with the effect of cyclosporin A, rapamycin and mycophenolate mofetil (MMF) in the same experimental setting for CD8 T cell proliferation (D).
E) Modified CML assay to compensate the reduced number of viable responder cells after the stimulation phase under the effect of ABT-737. The inhibitory effect of ABT-737 on cytotoxicity was completely compensated if the same number of viable treated cells was added to the ^{51}Cr-labelled target cells during the killing phase. Data from a CML in the full-MHC mismatched combination B6 to BALB/c are shown (no statistical difference between allogeneic control and ABT-737 1 uM adjusted in all dilutions of standard culture).
F) BM3.3 mice were treated with ABT-737 or vehicle during 5 days. Treatment efficacy was demonstrated by lymphocyte depletion in blood (FACS). Splenocytes from ABT-737 and vehicle treated mice did not differ in their proliferation capacity after 4 days of MLR against B6 lymphocytes, as shown by CFSE dilution.

days of MLR in a concentration dependent manner (data not shown). However, the small fraction of alloreactive CD8 T cells that survived exposure to ABT-737 (PI negative population) was not altered in its activation status (CD25 expression, Fig. 13C) and proliferation capacity (CFSE dilution, Fig. 13D, ABT-737 plots) even at high ABT-737 concentrations. Moreover, the cytotoxicity of surviving lymphocytes was not reduced compared to control, as shown in experiments, in which after activation under the effect of ABT-737 the number of viable cells was adjusted before starting the killing phase (Fig. 13E). The relevance of such observations was confirmed with an *ex vivo* experiment. After 5 daily injections of ABT-737 (50 mg/kg/day) BM3.3 mice presented marked lymphopenia in blood compared with control animals (Fig. 13F). The mice were sacrificed and their splenocytes used for MLR with irradiated B6 lymphocytes stimulators. Ex vivo CD8 T cell activation (data not shown) and proliferation (Fig. 13F) were not reduced by the previous exposure to ABT-737. Therefore, the immunosuppressive effect of ABT-737 appears to be explained by clone size reduction in the alloreactive lymphocyte population by apoptosis, sparing the physiological functions of remaining viable CD8 T cells. This contrasted with the mechanism of action of established small-molecule immunosuppressive drugs: in the same experimental setting CsA, rapamycin and mycophenolic acid directly suppressed proliferation, whereas ABT-737 reduced the alloreactive clone size (Fig. 13D).

ABT-737 is highly selective for lymphatic tissues.

The intrinsic apoptosis pathway is fundamental and universal in mammalian cells. Therefore, systemic toxicity might limit the clinical application of Bcl-2 inhibition as an immunosuppressive principle. It has been shown that the relevance of different apoptosis regulatory mechanisms varies among tissues, eventually determining the selectivity of different Bcl-2 inhibitors (Vogler, Dinsdale et al. 2009) and – as a consequence – the toxicity of the treatment. To assess the tissue selectivity of ABT-737 we analyzed by immunohistochemistry the number of apoptotic cells in different organs after injection of ABT-737 (50 mg/kg) or DMSO-containing vehicle. Six hours after injection, ABT-737 treated mice presented a 10-fold increase in the number of apoptotic cells in spleen and a 2-fold increase in the thymus compared to vehicle-treated controls (Fig. 14A-B). Parenchymal organs such as kidney, liver or heart were minimally affected by the treatment. To exclude a role of a possible variability in ABT-737 exposure among different organs, the selectivity of ABT-737 was further assessed by a specific analysis of epithelial and

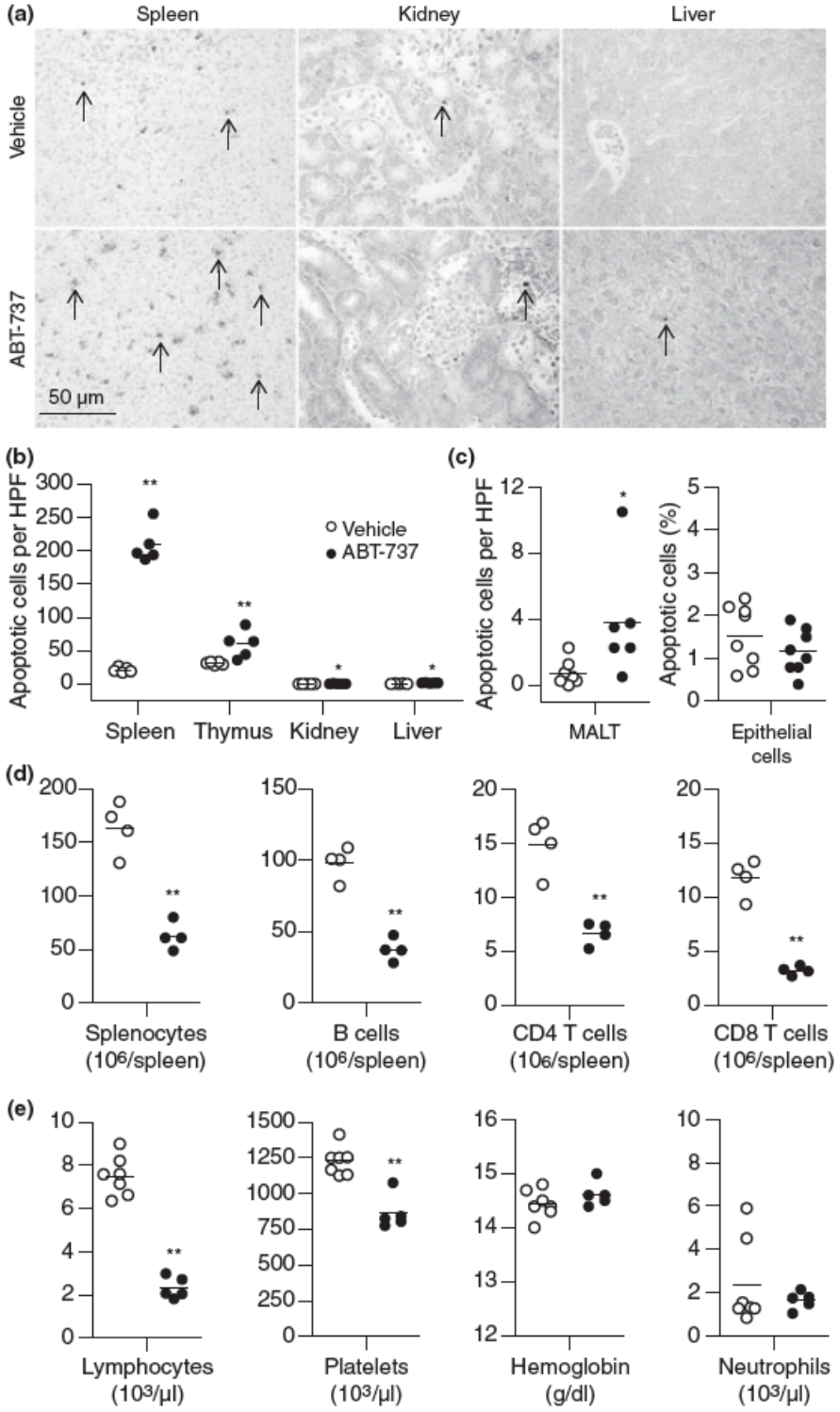

Fig. 14. The pro-apoptotic effect of ABT-737 is selective for lymphocytes.

A-B) B6 mice were sacrificed 6h after injection of ABT-737 (50 mg/kg) or vehicle. Apoptosis detection by immunohistochemistry for single-stranded DNA revealed a 10-fold increase in the number of apoptotic cells in the spleen and a 2-fold increase in the thymus. Kidney and liver tissue presented a low number of apoptotic cells and were minimally affected by the treatment (mean number of F7-26 positive cells per 10 high power fields [HPF], n = 5 mice/group; * $p<0.05$, ** $p<0.01$).

C) After 5 daily injections of ABT-737 (50 mg/kg/day) the intestine of B6 mice was harvested. Treatment with ABT-737 significantly increased the number of apoptotic cells in the mucosa associated lymphatic tissue (MALT) as shown by TUNEL staining (mean number of apoptotic cells per HPF), but had no impact on the frequency of apoptotic cells in isolated intestinal epithelial cells as measured in FACS (n = 6-9 mice/group, * $p<0.05$).

D) Analysis of splenocyte subpopulations by FACS after 5 daily injections revealed that B and T cells were similarly affected by ABT-737 (number of cells/spleen, n = 4 mice/group; ** $p<0.01$).

E) After 18 daily injections of ABT-737 (50 mg/kg/day) B6 mice presented a significant lymphopenia and a moderate thrombocytopenia compared to control animals. Erythrocytes and neutrophils were not affected by the treatment (n = 5-7 mice/group).

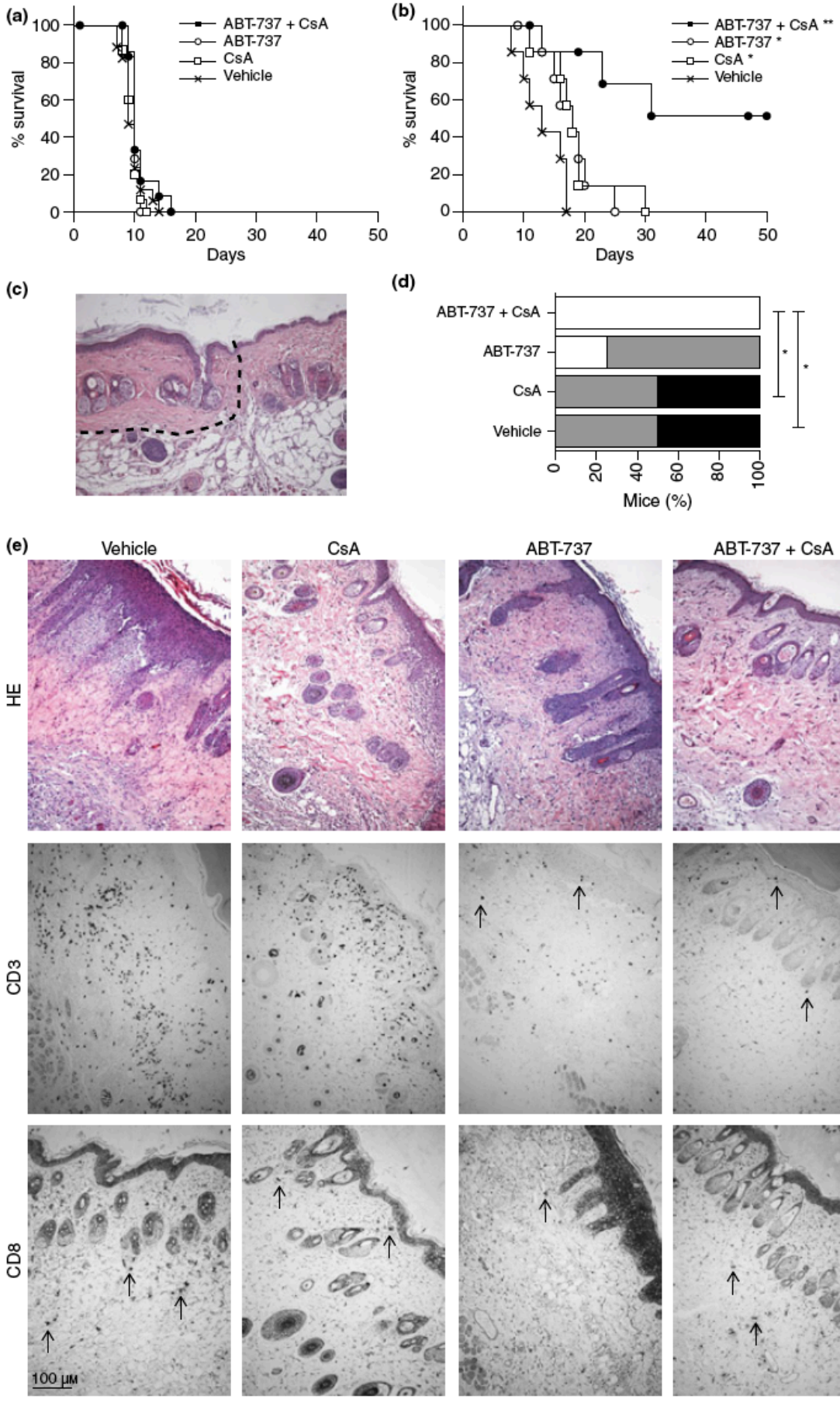
(a)
% survival
Days
ABT-737 + CsA
ABT-737
CsA
Vehicle
(b)
% survival
Days
ABT-737 + CsA **
ABT-737 *
CsA *
Vehicle
(c)
(d)
ABT-737 + CsA
ABT-737
CsA
Vehicle
Mice (%)
(e)
Vehicle
CsA
ABT-737
ABT-737 + CsA
HE
CD3
CD8
100 μm

Fig. 15. ABT-737 inhibits allospecific T cell responses *in vivo*.

A) In the fully MHC-mismatched combination BALB/c to B6 neither ABT-737 (50 mg/kg/day), nor CsA (10 mg/kg/day) or a combination of both prolonged skin graft survival (data pooled from two identical experiments, n=7-12 mice/group).

B-C) In the single MHC-mismatched model bm1 to B6 ABT-737 inhibited skin graft rejection synergistically with CsA (Log-rank test in comparison to the vehicle group, * $p<0.05$, ** $p<0.01$, n=7-8 mice/group). As a result, long-term graft survival was observed in 50% of the recipients in the combination group. Biopsies of the non-rejected skin grafts did not present any histological sign for rejection at day 50 after transplantation, as shown by the comparison of transplanted (left to the dashed line) and native skin (right to the dashed line) in one representative example.

D-E) Histological analysis of bm1 skin grafts at day 8 after transplantation onto B6 recipients confirmed a marked inhibition of T cell mediated rejection by ABT-737 in synergism with CsA. Representative examples of the morphological evaluation by routine histology (haematoxylin eosin staining, HE) and immunohistochemistry for CD3 and CD8 from mice treated with vehicle, CsA (10 mg/kg/day), ABT-737 (50 mg/kg/day) or a combination of both drugs are shown. Please note the prominent thickening of the epidermis and cellular infiltration in the vehicle treated mice (HE, upper left). A marked reduction of the CD3 positive infiltrating cells (arrows in second row, third and fourth column) could be demonstrated. The number of CD8 positive cells was low as compared to the CD3 positive cells (arrows in the lower panels). Semi-quantitative analyses for CD3 are presented in Fig. 15D according to a 3 degree score (white: no infiltration, gray: infiltration in less then 50% of the graft, black: infiltration in more then 50% of the graft; Fisher's exact test comparing no infiltration vs. any infiltration, * $p<0.05$; n = 3-4 mice/group).

lymphatic cells from one and same organ: the intestine. We found that after 5 daily injections of ABT-737 (50 mg/kg/day) B6 mice presented a significant increase in the number of apoptotic cells in the mucosa associated lymphatic tissue (Peyer's patches), but not in the epithelium (TUNEL staining). FACS analysis of isolated intestinal epithelial cells from colonic mucosa confirmed that ABT-737 did not increase the fraction of apoptotic cells in this tissue (Fig. 14C).

Spleen FACS analyses after 5 daily injections revealed that ABT-737 exposure resulted in a 60-65% reduction in the number of total splenocytes, and that different lymphocyte subpopulations were similarly affected by the treatment (Fig. 14D).

In addition, hematological analyses were performed. After 18 daily injections of ABT-737 (50 mg/kg/day) B6 mice showed a significant reduction of lymphocyte and platelet counts compared to vehicle-treated controls, but erythrocytes and neutrophils were not affected (Fig. 14E).

ABT-737 inhibits skin graft rejection synergistically with low-dose cyclosporine A.

The immunosuppressive effect of ABT-737 on T cell mediated rejection was assessed *in vivo* in a mouse skin graft model. In the fully MHC-mismatched combination BALB/c to B6 neither ABT-737 (50 mg/kg/day), nor low-dose CsA (10 mg/kg/day) or a combination of ABT-737 and low-dose CsA were sufficient to significantly prolong skin graft survival (Fig. 15A). However, in the MHC class I single antigen mismatched combination bm1 to B6, daily injections with ABT-737 from day 5 before transplantation until rejection significantly prolonged skin graft survival compared to vehicle control (median graft survival vehicle vs. ABT-737: 13 vs. 18 days, p=0.03, Fig. 15B). Notably, ABT-737 and CsA displayed a similar, significant but rather modest, effect as single agents in this model, but their immunosuppressive potency was markedly increased in combination: a long-term graft survival was achieved in 50% of recipients treated with ABT-737 (50 mg/kg/day, i.p.) and CsA (10 mg/kg/day, s.c.) from day 5 before transplantation (median graft survival CsA vs. ABT-737+CsA: 18 days vs. undefined, p=0.008; vehicle vs. ABT-737+CsA, p=0.001, Fig. 15B). Fifty days after transplantation recipients that had not rejected their grafts were sacrificed. The histological analysis revealed an almost complete absence of inflammatory infiltrates (Fig. 15C), supporting the hypothesis of a suppression of the allospecific immune response. Moreover, the immunosuppressive effect of ABT-737 and the synergistic effect

in combination with CsA were confirmed in an additional experiment, in which skin grafts were analyzed histologically 8 days after transplantation. At that time point a prominent reduction of CD3 positive T cells was seen in mice treated either with ABT-737 or the combination of ABT-737 and CsA (vehicle vs. ABT-737+CsA, p=0.03, Fig. 15D). CD8 positive T cells were low in number but also demonstrated a trend towards a reduced infiltration (Fig. 15E). Notably, syngeneic skin grafts were not affected by ABT-737 treatment with or without CsA (follow up of more than 150 days, data not shown), indicating that ABT-737 did not negatively interfere with the healing process after surgery.

ABT-737 inhibits allo-specific humoral responses.

The serum level of allo-specific antibodies was measured 15 days after transplantation of BALB/c skin grafts onto B6 recipients. Although not sufficient to prolong skin graft survival in this setting (Fig. 15A), ABT-737 significantly inhibited the production of allo-specific antibodies as measured by indirect FACS (vehicle vs. ABT-737, IgG, p=0.002, Fig. 16). Interestingly, also the B cell response was further inhibited in the group treated with a combination of ABT-737 (50 mg/kg/day) and low-dose CsA (10mg/kg/day). Similar results were obtained for IgG and IgM allo-specific antibodies (vehicle vs. ABT-737 + CsA, IgG p=0.001, IgM p=0.001; CsA vs. ABT-737 + CsA, IgG p=0.003, IgM p=0.028). Thus, ABT-737 potently inhibited allospecific T and B cell responses *in vivo* and displayed a marked synergistic effect with the calcineurin inhibitor CsA.

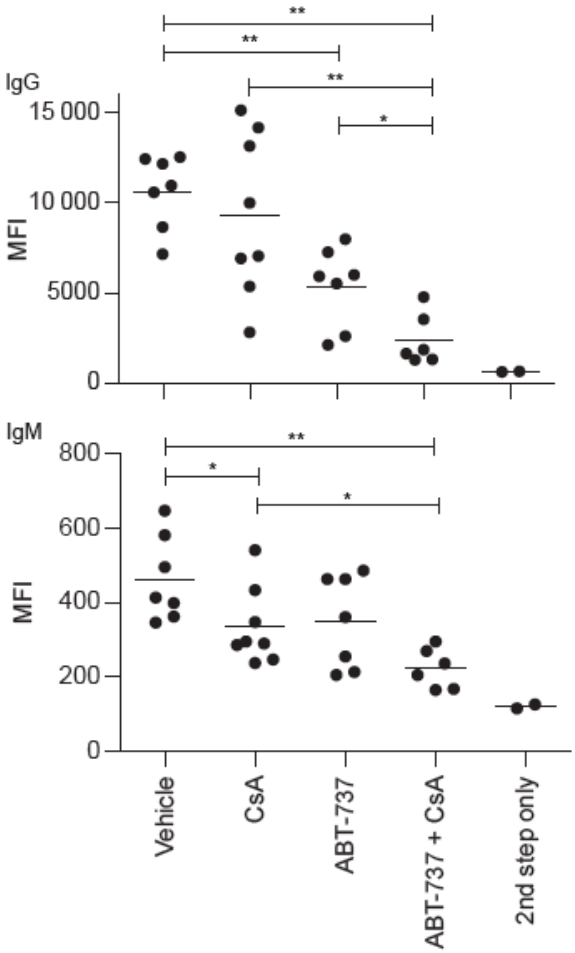

Fig. 16. ABT-737 inhibits allospecific B cell responses *in vivo*.

Serum levels of allospecific IgG and IgM antibodies were measured 15 days after skin transplantation in the BALB/c to B6 combination. ABT-737 (50 mg/kg/day) synergistically with CsA (10 mg/kg/day) inhibited allospecifc humoral response (MFI: mean fluorescence intensity; * p<0.05, ** p< 0.01, n = 6-8 mice/group). Representative results of one of two experiments are shown.

Discussion

BH3-mimetics represent a new class of drugs to inhibit detrimental immune responses such as in autoimmunity (Bardwell, Gu et al. 2009), allergy (Karlberg, Ekoff et al. 2010) and chronic inflammation. Here we report that ABT-737 potently suppressed allospecific T and B cell responses. The immunosuppressive effect of ABT-737 was markedly increased in combination with low-dose CsA.

In our *in vitro* and *ex vivo* models, immunosuppression by ABT-737 was a sole manifestation of clone size reduction in the alloreactive T cell population induced by apoptosis. Although T cell depletion represents a well established immunosuppressive approach (Halloran 2004), the mechanism of action described here differs from strategies in current clinical protocols. In contrast to depleting antibodies such as antithymocyte globulin or cytotoxic drugs such as cyclophosphamide, Bcl-2-antagonists interact with pathways physiologically regulating periph-

eral T cell deletion and offer opportunities to better understand and to modulate these fundamental mechanisms as a novel immunosuppressive approach.

The efficacy of this concept was confirmed *in vivo* in a skin transplantation model. As a single agent ABT-737 significantly prolonged skin graft survival, but was not sufficient to fully prevent rejection in a MHC class I mismatched model. As ABT-737 did not completely deplete lymphocytes *in vivo* (Fig. 14D), we speculate that a subpopulation of alloreactive lymphocytes survived the pro-apoptotic effect of ABT-737 and was responsible for graft rejection. In contrast, in combination with low dose CsA ABT-737 completely prevented rejection in 50% of the recipients, as shown in the histological analysis of the grafts at day 50 after transplantation (Fig. 15B-C). Because of the high immunogenicity of the skin it is not surprising that a pharmacological combination was required to completely exploit the immunosuppressive potential of ABT-737. Of particular interest, the addition of a Bcl-2 inhibitor increased the immunosuppressive potency of CsA at a dosage that by its own was not sufficient to prevent graft rejection. We hypothesize that in the setting of a reduced clone size by ABT-737 a lower concentration of CsA was sufficient to control the pool of alloreactive T cells. This hypothesis is supported by the fact that the combination therapy was not sufficient to inhibit the larger pool of allo-reactive cells in the fully-MHC mismatched combination. However, additional interactions between the calcineurin and the intrinsic apoptosis pathways have been previously described and might play a role in this setting (Shibasaki, Kondo et al. 1997).

Bcl-2 inhibitors suppress immune responses through a novel pharmacological target and open new options for pharmacological combinations. Because of the favorable synergism with CsA, ABT-737 might find a clinical application as part of a low dose calcineurin inhibitor (CNI) based regime and help reducing CNI long-term toxicity. Moreover, based on its well-established antineoplastic properties, we expect that ABT-737 would reduce the incidence of tumors in transplant recipients. This aspect would differentiate Bcl-2 inhibitors from most current immunosuppressive drugs, and might have a major impact on survival in this high-risk situation for cancer development.

Apoptosis is a fundamental cellular mechanism, and tissue selectivity of Bcl-2 inhibitors is crucial to limit systemic toxicity. Although a detailed analysis of the mechanisms determining tissue selectivity of ABT-737 was beyond the objectives of this study, our experiments showed that ABT-737 is selective for lymphatic cells and tissues. These results, which are supported by data from clinical trials using the ABT-737 orally bioavailable counterpart ABT-263 (Wilson, Tulpule et al. 2007), suggest a favorable toxicity profile for ABT-737. Further analyses are required for a better understanding of the regulation of Bcl-2 proteins in inflamed tissues and to better assess the selectivity of ABT-737 after surgery. In this context, the normal survival and wound healing of syngeneic grafts in ABT-737 treated mice represent a promising result, thereby distinguishing ABT-737 from anti-proliferative drugs such as mTOR inhibitors.

Currently available therapeutical options to control humoral rejection are limited. ABT-737 as a single agent and particularly in combination with CsA suppressed the alloantibody response in a fully MHC mismatched skin graft model. Although our mechanistic studies primarily focused on T cell responses, we observed that B and T cells were similarly depleted by ABT-737 (Fig. 14D). Therefore, we suggest B cell depletion as the principal explanation for this effect of ABT-737, but a concomitant T cell help inhibition is likely to be involved and may explain the synergistic effect with CsA. Further studies are required to better assess the effect of ABT-737 on plasma cells.

Thus, our data strongly support further evaluation of Bcl-2 inhibitors as novel class of immunosuppressive drugs with potential broad clinical application in the field of allotransplantation.

Acknowledgments
We thank A.-M. Schmitt-Verhulst for providing the BM3.3 mouse strain, K. Leucht and M. Krebs for intestinal cell isolation, and K. Bruni and her team for the blood analyses.

Chapter 4: Resistance to ABT-737 in activated T lymphocytes: molecular mechanisms and reversibility by inhibition of the calcineurin-NFAT pathway

Pietro E. Cippà[1,2], Anna K. Kraus[1,2], Maja T. Lindenmeyer[1,2], Jin Chen[1,2], Annick Guimezanes[3], Philip D. Bardwell[4], Thomas Wekerle[5], Rudolf P. Wüthrich[1,2], Thomas Fehr[1,2]

[1] Institute of Physiology, University of Zürich, Switzerland
[2] Division of Nephrology, University Hospital Zürich, Switzerland
[3] Centre d'Immunologie de Marseille-Luminy, Université de la Méditerranée, Marseille, France
[4] Abbott Bioresearch Center, Worcester, USA
[5] Division of Transplantation, Department of Surgery, Medical University of Vienna, Vienna, Austria

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Abstract
Dynamic regulation of the intrinsic apoptosis pathway controls central and peripheral lymphocyte deletion and may interfere with the pro-apoptotic potency of Bcl-2 inhibitors such as ABT-737. By following a T cell receptor-transgenic population of alloantigen-specific T cells, we found that sensitivity to ABT-737 radically changed during the course of allo-specific immune responses. Particularly, activated T cells were fully resistant to ABT-737 during the first days after antigen recognition. This phenomenon was caused by a T cell receptor-calcineurin-NFAT-dependent up-regulation of A1 and was therefore prevented by cyclosporine A. As a result, exposure to ABT-737 after alloantigen recognition induced selection of alloreactive T cells *in vivo*, whereas in combination with low-dose cyclosporine A ABT-737 efficiently depleted allo-reactive T cells in mouse host-versus-graft and graft-versus-host models. Thus, ABT-737 resistance is not a prerogative of neoplastic cells, but it physiologically occurs in T cells after antigen recognition. Reversibility of this process by calcineurin inhibitors opens new pharmacological opportunities to modulate this process in the context of cancer, autoimmunity and transplantation.

Introduction

Bcl-2 inhibition represents a novel pharmacological principle to control lymphoid malignancies and detrimental immune responses (Vogler, Dinsdale et al. 2009; Carrington, Vikstrom et al. 2010). Of particular scientific and potential clinical interest are the small-molecule Bcl-2 inhibitors ABT-737 and its bioavailable counterpart navitoclax (ABT-263) (Oltersdorf, Elmore et al. 2005; Tse, Shoemaker et al. 2008). ABT-737 binds with high affinity to the anti-apoptotic Bcl-2 family members Bcl-2, Bcl-xL and Bcl-w. By this mechanism it prevents them from sequestering pro-apoptotic BH3 proteins and therefore indirectly initiates the apoptotic cascade. In contrast, ABT-737 has a low affinity to Bcl-2-A1 (A1, Bfl-1), Mcl-1 and Bcl-B. These particular molecular binding characteristics may be important for tissue selectivity and - as a consequence – for the favorable toxicity profile of ABT-737 and ABT-263 (Wilson, Tulpule et al. 2007), but limits their therapeutic potency on lymphoma cells expressing A1 and Mcl-1 (Del Gaizo Moore, Schlis et al. 2008; Yecies, Carlson et al. 2010).

The physiological regulation of apoptosis in lymphocytes has been extensively investigated (Marsden and Strasser 2003) and may assume a new relevance in the context of therapeutic approaches selectively targeting Bcl-2 proteins. Focusing on the T cell compartment, it has been shown that the fate of a T cell is linked to the expression of a functional T cell receptor (TCR) (Cho, Kim et al. 2010) and its interaction with antigen presenting cells (APCs). The combination of signals through the TCR, co-stimulatory molecules (such as CD28 and 4-1BB) and cytokines (such as IL-2 and IL-15) dynamically modulates the intrinsic and the extrinsic apoptosis pathway in T lymphocytes and eventually controls central and peripheral T cell selection (Wojciechowski, Tripathi et al. 2007; Fischer, Belz et al. 2008; Hughes, Belz et al. 2008; Sabbagh, Pulle et al. 2008; Bouillet and O'Reilly 2009; Fehr, Lucas et al. 2010). Of particular interest are previous reports on a TCR-dependent up-regulation of A1 in the early phase after antigen recognition, which protects activated thymocytes and splenocytes from apoptosis without interfering with cell proliferation (Gonzalez, Orlofsky et al. 2003; Verschelde, Walzer et al. 2003). These mechanisms are crucial for the development and the maintenance of a functional immune system (Hughes, Belz et al. 2008) and might be influenced by drugs targeting the apoptosis pathway.

This hypothesis is supported by previous reports about the immunomodulatory properties of ABT-737 in several experimental models: ABT-737 had a beneficial effect on autoimmunity (Bardwell, Gu et al. 2009) and significantly inhibited solid allograft rejection in the mouse (Carrington, Vikstrom et al. 2010; Cippa, Kraus et al. 2011). However, immunosuppression by ABT-737 in a collagen-induced arthritis model was only effective in a preventive setting, but not in mice with established disease (Lawlor, Smith et al. 2011). Furthermore, the immunosuppressive effect of ABT-737 in a mouse skin graft model was rather limited as a single agent, but markedly increased in combination with cyclosporine A (CsA) (Cippa, Kraus et al. 2011). These data suggest that the pro-apoptotic potency of ABT-737 on lymphoid cells is altered in the context of inflammation and T cell activation.

Thus, in this study we examined the effect of ABT-737 on alloreactive T cells in the setting of host-versus-graft and graft-versus-host immune reactions. We found a unique selectivity profile of ABT-737 on T lymphocytes over the course of the immune response as a result of a transient, calcineurin-, NFAT- and A1-dependent resistance to ABT-737 after antigen recognition. The calcineurin inhibitor CsA blocked A1 up-regulation and prevented resistance to ABT-737 in activated T cells, thereby offering new options for effective combination therapies.

Material and Methods

Mice. C57BL/6 (B6, H-2^b), CBA (H-2^k), (CBAxB6)F1 (F1, H-$2^{b/k}$) and BM3.3 (CBA, H-2^k) mice were housed in specific pathogen-free conditions at the University of Zürich. The BM3.3 mouse (Auphan, Curnow et al. 1994), which expresses on all CD8 T cells a transgenic T cell receptor (TCR) selective for a naturally processed octapeptide bound to the allogeneic MHC class I molecule H-$2K^b$, was kindly provided by A.-M. Schmitt-Verhulst (Guimezanes, Barrett-Wilt et al. 2001). All animal experiments were performed according to protocols approved by the legal authority (Veterinary Office, Canton of Zürich, Switzerland).

Synchimeras and graft-versus-host reaction model. Synchimeric animals were generated as previously described (Fehr, Takeuchi et al. 2005; Haspot, Fehr et al. 2008). Briefly, $5x10^6$ bone marrow cells from BM3.3 mice were transplanted into naive CBA mice irradiated with 3 Gy on the same day. After 6-10 weeks, B6 splenocytes were injected and treatment with ABT-737 (50 mg/kg/d i.p.) or vehicle was started according to the experimental protocol. Donor-reactive BM3.3 CD8 T cells were monitored in blood using the Ti98 antibody, which selectively binds to the BM3.3 TCR (Buferne, Luton et al. 1992).

Graft-versus-host (GvH) reactions were studied in a parent to F1 model. F1 mice were generated by breeding CBA females and B6

males and expressed H-2^k and H-2^b. After adoptive transfer of 20-25x10^6 BM3.3 splenocytes (H-2^k background and therefore not rejected by the host, and selectively reacting against H-$2K^b$) GvH reactive cells were analyzed in the spleen using the Ti98 antibody.

Fluorescence activated cell sorting (FACS). FACS analyses were performed with a BD-FACSCanto (Becton Dickinson, Basel, Switzerland). Anti-mouse CD3-FITC, CD4-PE, CD8-APC, and propidium iodide (PI) were purchased from eBioscience (Frankfurt, D), anti-mouse CD25-PE/Cy7 from Biolegend (Uithoorn, NL). The Ti98 antibody was kindly provided by A.-M. Schmitt-Verhulst (Buferne, Luton et al. 1992). A secondary PE rat anti-mouse IgG was purchased from Becton Dickinson (Basel, CH).

Mixed lymphocyte reaction (MLR). MLR were performed in 96 wells plates with responder splenocytes stimulated by T cell-depleted (or CD8 T cell-depleted) splenocytes from allogeneic and syngeneic mice at a final concentration of 4 x10^6 cells/ml in RPMI medium containing, 10% fetal bovine serum, penicillin 100 U/ml, streptomycin 100 μg/ml, 2-mercaptoethanol 50 μM. Splenocytes were sorted by automatic magnetic cell separation using an autoMACS pro separator according to the protocols of Miltenyi Biotec (Bergisch Gladbach, D) to allow a selective analysis of responder CD4 and CD8 T cells in FACS.

Reagents and Drugs. ABT-737 was provided by Abbott Bioresearch (Worcester, USA): for *in vitro* experiments ABT-737 was dissolved in DMSO at a concentration of 5 mM and then diluted in culture medium. For *in vivo* applications ABT-737 was dissolved in polyethylene glycol, Tween 80, dextrose solution and DMSO and injected intra-peritoneally (i.p.) at 50 mg/kg/day. Antimycin A, CsA, Rapamycin and Tacrolimus were purchased from EnzoBiochem (Farmingdale, New York, USA), Cycloheximide from Sigma-Aldrich (Buchs, CH), VIVIT-R from Calbiochem (Merck, Darmstadt, D), Obatoclax (GX15-070) from Selleck (Houston, USA). CTLA4Ig (abatacept) was provided by Bristol-Myers Squibb. The anti-CD154 (CD40L) antibody MR1 was purchased from BioXCell (West Lebanon, USA). For *in vivo* application CsA was dissolved in ethanol and cremaphor EL (Sigma-Aldrich), then diluted in PBS and injected subcutaneously (s.c.).

Quantitative RT-PCR (qRT-PCR). Reverse transcription and qPCR were performed as reported earlier (Lindenmeyer, Eichinger et al. 2010). Pre-developed TaqMan reagents were used for mouse Bcl-2 (Mm00477631_m1), Bcl-XL (Mm00437783_m1), Mcl-1 (Mm01257352_g1) and for the housekeeper gene 18S rRNA (Applied Biosystems Europe, Rotkreuz, CH). For mouse A1, the following oligonucleotide primers and probe were designed to simultaneously detect Bcl-2A1a, Bcl-2A1b and Bcl-2A1d: sense primer 5'-ATG GAG GTT GGG AAG ATG G-3', antisense primer 5'-GAG CCA AGG TTC TCT CTG GTC-3', fluorescence labeled probe (FAM) 5'-GGC TGG CTG ACT TTT CTG CAG ATG A-3'. The expression of candidate genes in alloantigen-stimulated cells of culture was normalized by 18S rRNA and compared with syngeneically stimulated cells.

Western blot. For Western blot allo- and syngeneically stimulated lymphocytes were harvested with Ripa buffer and complete protease inhibitor cocktail (Roche, Mannheim, D) at different time points after stimulation *in vitro*. Extracted proteins were boiled in loading buffer for 5 minutes, resolved by 15% SDS-PAGE and transferred to an Immobilon-P membrane (Millipore, Eschborn, D). Membranes were blocked overnight with Tris-buffered saline (TBS)/5% fat-free skim milk, then incubated with a polyclonal anti-A1 antibody (Cell Signaling Technology, Danvers, USA) diluted 1:1000 overnight at 4°C and rinsed with TBS that contained 0.1% Tween 20. For detection a HRP-linked goat anti-rabbit antibody (1:4000, 30 min at room temperature; Cell Signaling Technology, Danvers, USA) and enhanced chemiluminescence substrate (Proteinsimple, Santa Clara, USA) were used. Membranes were also probed with anti-actin antibody (A2066, 1:1000, Sigma-Aldrich) as internal loading control.

Statistics. Student t-test, Mann-Whitney test and paired t-test were used to compare values between groups as appropriate. IC50-values were calculated using a log(inhibitor) vs. response model. P<0.05 was considered significant. Graph Pad Prism Software Version 5.0 was used for calculations.

Results

Activated T cells are resistant to ABT-737

To investigate the impact of allogeneic T cell activation on the sensitivity to the Bcl-2 inhibitor ABT-737, we used the transgenic mouse strain BM3.3, which expresses on all CD8 T cells a transgenic TCR specific for the

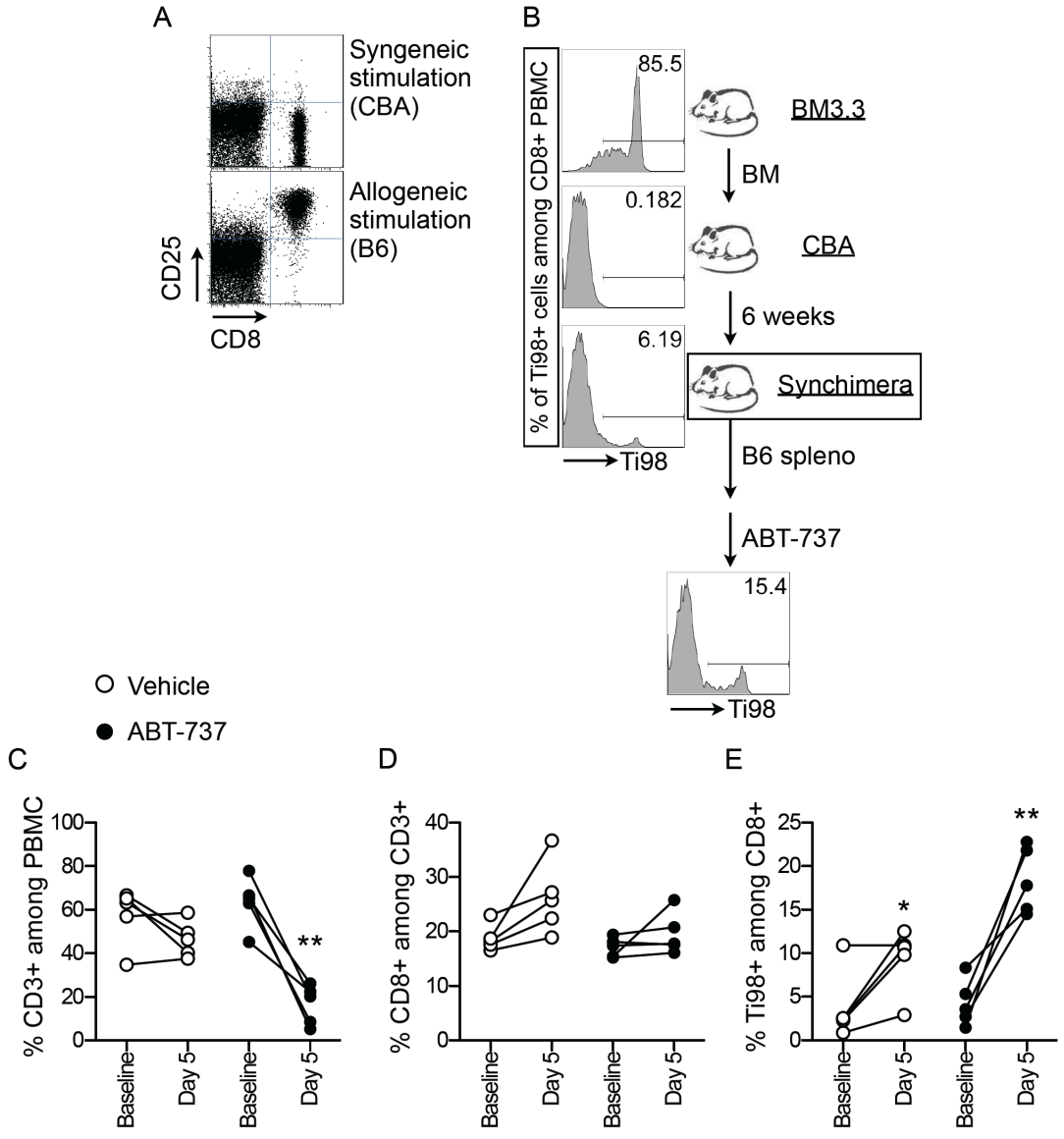


Fig. 17. Allo-reactive CD8 T cells are resistant to ABT-737 after donor specific transfusion

(A) Characterization of the BM3.3 model: after 48h of MLR with BM3.3 responders and CD8-depleted B6 stimulators all responder (Ti98+) CD8 T cells were activated, as measured by CD25 expression in FACS. (B) Experimental setup: Synchimeric mice were generated by bone marrow (BM) transplantation from BM3.3 mice into CBA recipients after non-lethal total body irradiation (3Gy). After 6 weeks, synchimeras expressed the BM3.3 TCR (Ti98+) on about 6% of the whole CD8 positive population. The mice were primed by i.v. injection of B6 splenocytes, and two days later treatment with ABT-737 was started. (C-D) Exposure to ABT-737 induced a relative and absolute reduction of CD3+ cells in peripheral blood and similarly affected CD4+ and CD8+ T cells. (E) The increase of the percentage of Ti98+ cells among CD8 T cells was significantly higher in mice exposed to ABT-737 ($p<0.01$). Statistical comparison of data registered at baseline and at day 5 by paired t-test; *$p<0.05$, **$p<0.01$; n=5. Representative results of one of two independent experiments are shown.

MHC class I molecule H-2K^b and can be detected by the clonotypic antibody Ti98. In a first experiment we transplanted BM3.3 bone marrow (BM) into non-lethally irradiated CBA mice in order to create synchimeric mice that express the BM3.3-TCR only on a fraction of the CD8 T cell pool. This well-defined homogeneous population of alloreactive CD8 T cells could then be followed during the course of a host-versus-graft (HvG) response in the context of an otherwise physiological immune system (Fig. 17A-B). Synchimeras received a donor specific transfusion (20x10^6 B6 splenocytes, i.v.,

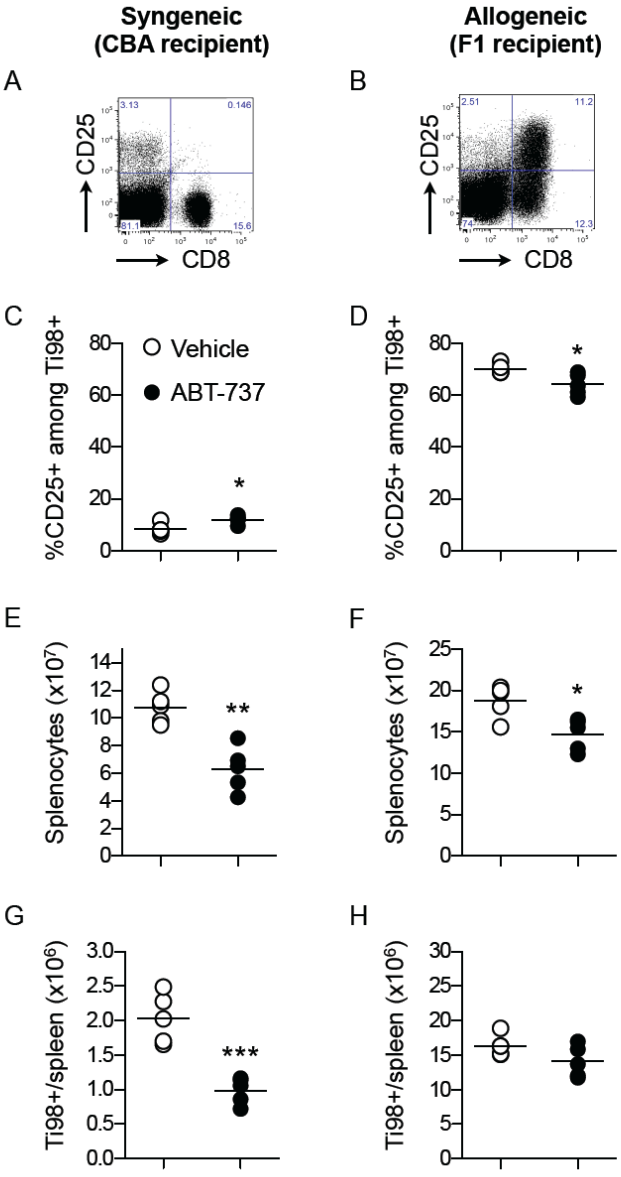


Fig. 18. Activated CD8 T cells are resistant to ABT-737 in a graft-versus-host model

BM3.3 splenocytes were adoptively transferred to CBA (syngeneic) or (CBAxB6)F1 (allogeneic) recipients to assess the effect of ABT-737 on GvH reactive cells. After 3 daily injections of ABT-737 (filled circles) or vehicle (open circles), splenocytes were analyzed by FACS. (A-B) Allogeneic CD8 T cell activation was confirmed by CD25 expression and (B-C) a selective analysis of Ti98+ cells indicated that ABT-737 did not substantially influence this process. (E-F) A similar reduction in the number of total splenocytes was registered in both groups (about 30% reduction, please note the different total number of splenocytes in different recipient strains). (G-H) The total number of Ti98 positive cells decreased in the syngeneic, but not in the allogeneic combination, indicating resistance to ABT-737 in allo-activated CD8 T cells. Statistical comparison ABT-737 vs. vehicle; *p<0.05, **p<0.01, ***p<0.001; n=5. Representative results of one of two independent experiments are shown.

DST) and treatment with either ABT-737 (50 mg/kg/day) or vehicle, starting at day 2 after DST. At day 5 after priming, mice treated with ABT-737 presented a 75% reduction of T cells in peripheral blood (Fig. 17C); CD4 and CD8 T cells were similarly affected by the treatment (Fig. 17D). After antigen recognition the percentage of Ti98 positive cells among CD8 T cells increased in both groups, but this effect was markedly enhanced in the ABT-737 group compared to control (Fig. 17E). This observation is explained by a selection of activated Ti98+ cells among CD8 T cells under the effect of ABT-737, which may be further enhanced by homeostatic proliferation in a lymphopenic environment.

To limit the confounding effect of homeostatic proliferation we performed an analogous experiment in a GvH model. The combination of a parent to F1 model with the BM3.3 transgenic system allowed us to specifically analyze a homogeneous population of host-reactive CD8 T cells in the absence of rejection by the recipient and without the effect of any conditioning regime (such as total body irradiation), that may alter the immune response and apoptosis regulation. We minimized the effect of T cell proliferation by choosing a short protocol: BM3.3 splenocytes were adoptively transferred into (CBAxB6)F1 (allogeneic stimulation) or CBA recipients (syngeneic control). On day 1 after transfer treatment with ABT-737 (50 mg/kg/day) or vehicle was started, and two days later recipient mice were sacrificed for FACS analysis. ABT-737 minimally influenced the activation of Ti98 cells (Fig. 18C and D) and similarly reduced the number of total splenocytes in F1 and CBA recipients by about 30% reduction (Fig. 18E and F). However, in the syngeneic combination, Ti98+ cells were similarly reduced as total T cells and total splenocytes (Fig. 18G), whereas in F1 recipients alloactivated donor-reactive CD8+Ti98+ cells were resistant to ABT-737 (Fig. 18H). As a result, the total number of Ti98+ cells in CBA recipients was markedly reduced after ABT-737 treatment, but no difference in the total number of Ti98 positive cells between the two groups was registered after allogeneic stimulation (Fig. 18G and H).

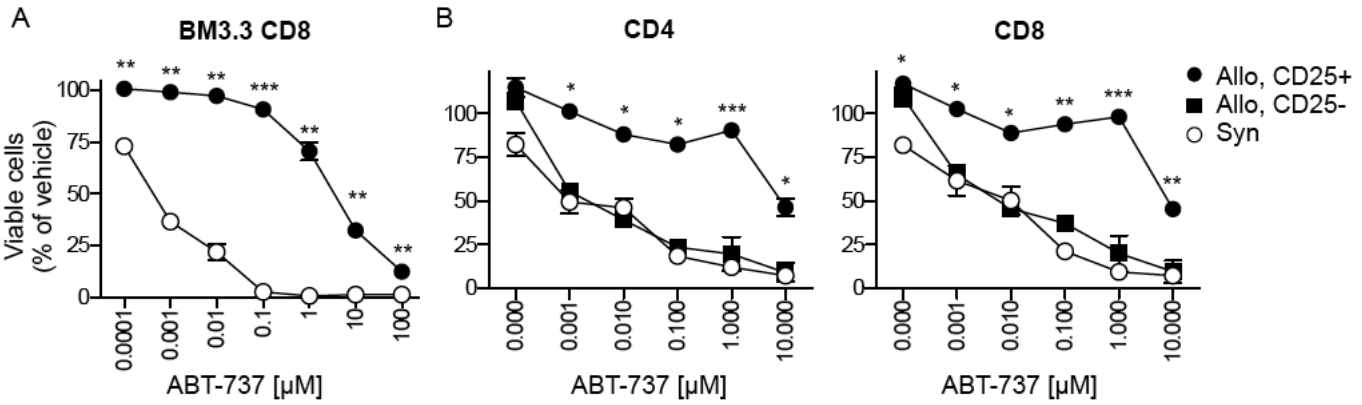
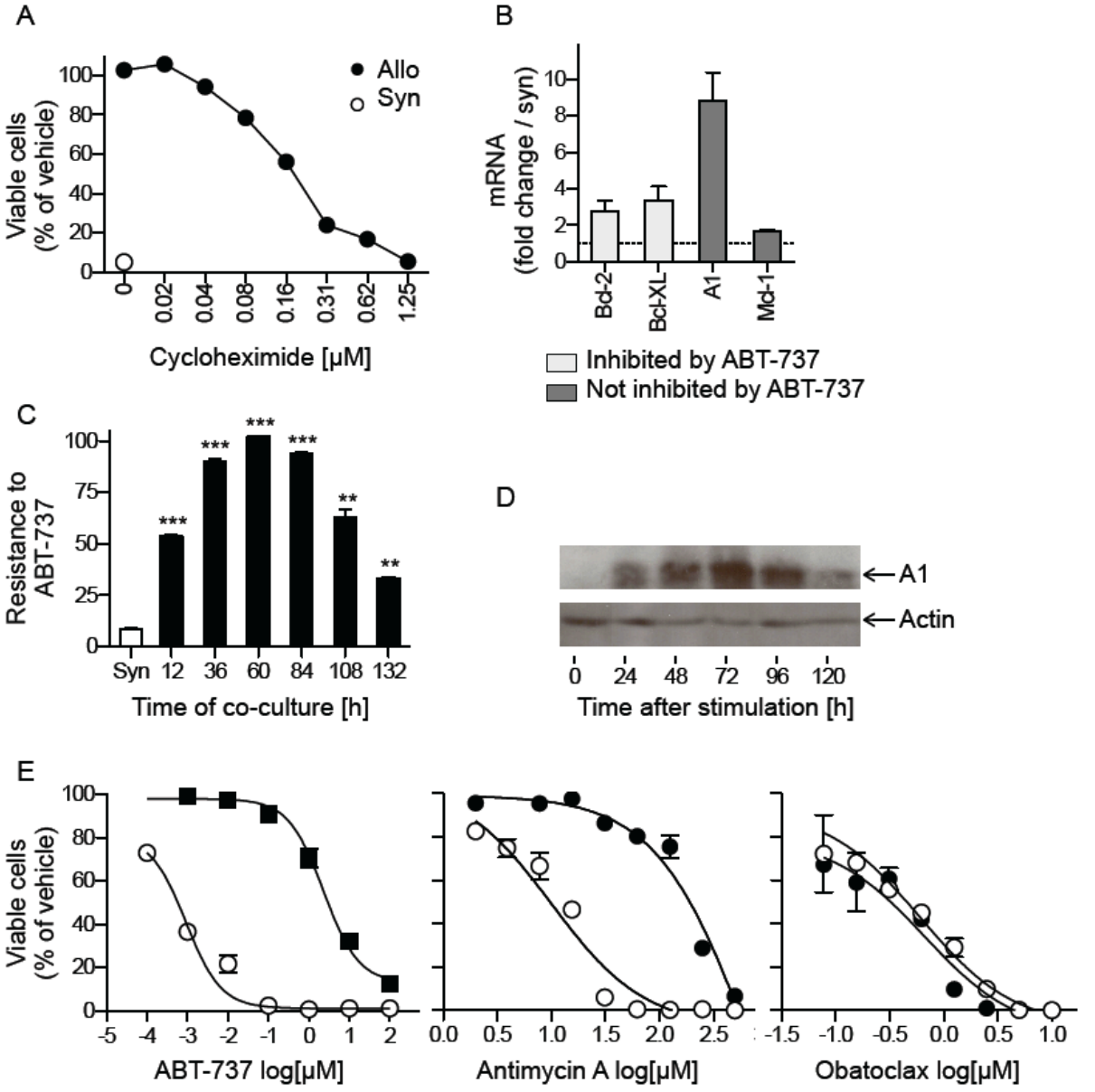


Fig. 19. Allo-activated T cells are resistant to ABT-737 *in vitro*

(A) BM3.3 splenocytes were stimulated with CD8-depleted B6 (allogeneic, filled circles) or CBA (syngeneic, open circles) splenocytes during 48h MLR and then treated with ABT-737 during additional 12h of MLR. Much higher concentrations of ABT-737 (1'000- to 10'000-fold) were required to induce apoptotis in allo-activated CD8 T cells compared to non-activated T cells. (B) Similarly, B6 splenocytes were stimulated with T cell-depleted CBA splenocytes during 48h MLR and then treated with ABT-737 during additional 12h of MLR. FACS analysis of responder CD4 and CD8 T cells revealed that activated (CD25+, filled circles) cells were resistant to ABT-737 compared to non-activated (CD25-, filled squares) cells in the same culture and also with syngeneically stimulated cells (open circles). Cell viability was assessed by PI exclusion in FACS in at least 3 independent experiments. Percentage of cells treated with vehicle is given. Statistical comparison of allo vs. syn: *p<0.05, **p<0.01, ***p<0.001.

These data strongly suggest that resistance of activated Ti98+ T cells to ABT-737 had developed in both HvG and GvH experiments.

This hypothesis was further tested *in vitro* in a mixed lymphocytes reaction (MLR) model. BM3.3 splenocytes were cultured with CD8-depleted allogeneic B6 or syngeneic CBA splenocytes during 48h and then treated with ABT-737 for additional 12h. Cell viability analysis by propidium iodide exclusion in FACS revealed that a 1'000- to 10'000-fold higher concentration of ABT-737 was required to induce apoptosis in CD8 T cells



Bcl-2 inhibitor	Low affinity to	IC50 Syn (95% CI)	IC50 Allo (95% CI)
ABT-737	A1, Mcl-1	0.0009 (0.00085-0.00099)	2.32 (1.67-3.28)
Antimycin A	A1	10.09 (5.04-20.20)	685.7 (232.4-2023)
Obatoclax (GX15)	- (pan-inhibitor)	0.61 (0.39-0.95)	0.66 (0.27-1.60)

Fig. 20. *Up-regulation of A1 is crucial for resistance to ABT-737*

(A) BM3.3 splenocytes were stimulated with CD8 T cell-depleted splenocytes from B6 (allo) or CBA (syn) donors during 24h of MLR under different concentration of cycloheximide and then treated with ABT-737 (1 uM) or vehicle. Alloantigen-stimulated cells without cycloheximide were resistant to ABT-737, but cycloheximide prevented this process in a concentration-dependent manner. (B) After 6h of MLR the expression of Bcl-2 family genes was analyzed in BM3.3 CD8 cells by qRT-PCR: we registered a 9-fold up-regulation in the expression of A1 and a small increase of Bcl-2 and Bcl-xL in comparison to syngeneically stimulated cells. (C) For a time course analysis BM3.3 splenocytes were stimulated for up to 6 days with B6 or CBA splenocytes and then treated with ABT-737 for additional 12h. Resistance to ABT-737 dynamically changed over time after allogeneic stimulation, reaching a maximum 2-3 days after stimulation and rapidly declining thereafter, whereas syngeneically stimulated cells remained sensitive to ABT-737 over time (one representative time point is shown). Cell viability of BM3.3 CD8 T cell was assessed by PI exclusion in FACS in at least 3 independent experiments. Percentage of cells treated with DMSO-containing vehicle is given. Statistical comparison of allo vs. syn: *p<0.05, **p<0.01, ***p<0.001. (D) A parallel analysis of A1 by Western blot revealed that ABT-737 resistance and A1 expression strongly correlated over time. (E) The role of different anti-apoptotic Bcl-2 factors in determining resistance to ABT-737 was investigated comparing the pro-apoptotic effect of ABT-737, antimycin A and Obatoclax on activated (filled circles) and non-activated (open circles) BM3.3 CD8 T cells. T cell activation induced resistance to ABT-737 and to antimycin A but not to Obatoclax, demonstrating a crucial role of A1 in this phenomenon. Percentage of cells treated with DMSO-containing vehicle is given. Half maximal inhibitory concentration (IC50) and 95% confidence interval (95% CI) are reported in the table.

after allogeneic stimulation (Fig. 19A). To exclude a transgenic artifact the same experiment was repeated with B6 responders and T-cell depleted CBA stimulators. Activated (CD25+) CD8 T cells were much more resistant to ABT-737 compared to non-activated (CD25-) cells in the same culture and to syngeneically stimulated (non-activated) T cells. The same phenomenon was observed for CD4 T cells (Fig. 19B). Thus, T cell activation induces resistance to ABT-737 *in vitro* and *in vivo*.

Molecular mechanism of resistance to ABT-737 in activated T cells

The regulation of apoptosis is complex, and several mechanisms may be involved in resistance to ABT-737 after T cell activation. In MLR experiments using the BM3.3 system, we found that exposure to the ribosome blocker cycloheximide during the stimulation phase prevented the establishment of ABT-737 resistance, indicating that protein synthesis was required to induce this anti-apoptotic state (Fig. 20A). Previous studies in tumor models revealed that the expression of anti-apoptotic Bcl-2 proteins with a low binding affinity to ABT-737, such as A1 and Mcl-1, resulted in resistance to this compound (Al-Harbi, Hill et al. 2011). Therefore, we first assessed the impact of T cell activation on the expression of various anti-apoptotic Bcl-2 proteins in our system. Analyses by qRT-PCR revealed that T cell activation rapidly influenced the gene expression pattern of Bcl-2 family members (Fig. 20B): among the Bcl-2 members that are not inhibited by ABT-737, expression of A1 was 9-fold higher in alloantigen-stimulated than in non-activated cells. In contrast, expression of Mcl-1 did not change. When looking at time kinetics we found that resistance to ABT-737 is a transient phenomenon: it rapidly develops after T cell stimulation, but progressively vanished after 4-5 days of culture (Fig. 20C). This evolution strongly correlated with expression of A1 protein over time (Fig. 20D), supporting the hypothesis of a crucial role of this particular factor. A selective inhibition of A1 in mouse cells is complicated due to the presence of 4 homologous genes for A1 in the mouse genome. Just one of them – A1-a – was successfully targeted in a knock-out mouse (Hamasaki, Sendo et al. 1998) and selective pharmacological A1 inhibitors are currently not available (Cashman, MacDonald et al. 2010). Therefore, we applied a reversed approach using different Bcl-2 inhibitors with a defined binding profile. We found that T cell activation induced resistance to Bcl-2 inhibition by ABT-737 (no binding of A1 and Mcl-1) and by Antimycin A (no binding of A1 only), but had no impact on the pro-

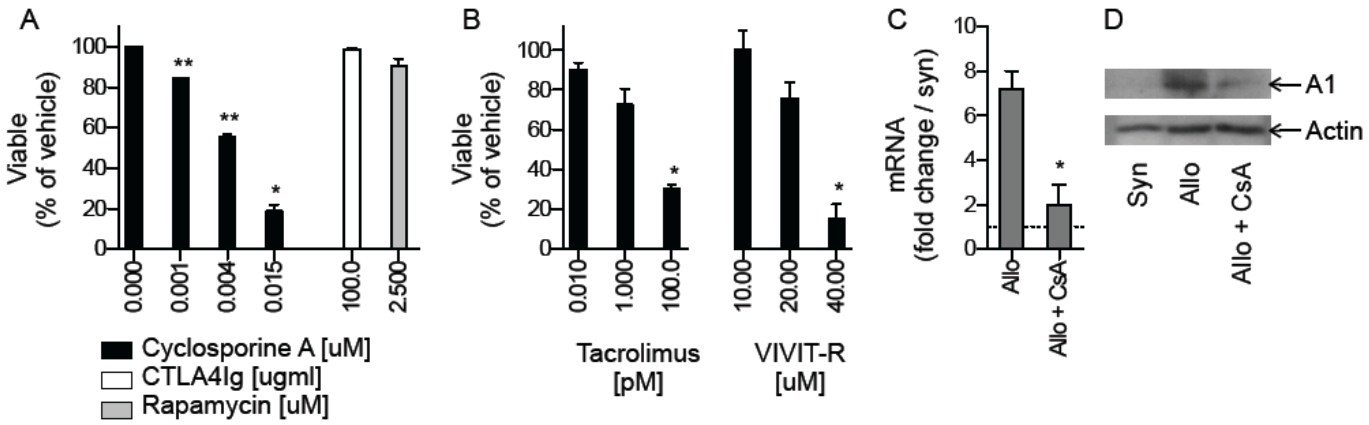


Fig. 21. Signal 1 determines resistance to ABT-737 in activated T cells

(A) Selective inhibitors of signal 1, 2 and 3 were added to an MLR during the stimulation phase with BM3.3 splenocytes reacting against CD8 T cell-depleted B6 splenocytes to investigate the role of the different T cell activation pathways for resistance to ABT-737 (1 uM, during additional 12h of culture). The calcineurin inhibitor and signal 1 blocker cyclosporine A prevented resistance to ABT-737 in a concentration-dependent manner, whereas inhibition of signal 2 by CTLA4Ig and signal 3 by rapamycin did not influence this process. (B) The results obtained with CsA were confirmed by other inhibitors of this same pathway, namely the alternative calcineurin inhibitor tacrolimus and the NFAT-inhibitor VIVIT-R. Cell viability of BM3.3 CD8 T cells was assessed by PI exclusion in FACS in at least 3 independent experiments. Percentage of cells treated with vehicle is given. Statistical comparison of ABT-737 vs. vehicle: *p<0.05, **p<0.01. (C, D) Exposure to CsA during the stimulation phase inhibited the up-regulation of A1 as assessed by qRT-PCR (C) and Western blot (D).

apoptotic potency of the pan-Bcl-2 inhibitor Obatoclax (Fig. 20E). Thus, A1 up-regulation is the crucial factor determining resistance to ABT-737 in activated T cells.

T cell activation signaling and resistance to ABT-737

According to the 3-signal concept, physiological T cell activation is determined by the concurrent stimulation of the TCR (signal 1) together with a costimulatory signal through CD28 and CD154 (signal 2) and by the effect of cytokines such as IL-2 and IL-15 (signal 3) (Matzinger and Kamala 2011). The link between resistance to ABT-737 and the different pathways involved in T cell activation was investigated dissecting the T cell activation process by blockade of different pathways during the stimulation phase (24h). We found that resistance to ABT-737 was prevented by blocking signal 1 with the calcineurin inhibitor CsA. In contrast, blocking of CD28 signaling by CTLA4Ig or of CD40 signaling by MR1 or using CD40-knock out stimulators (data not shown), and blocking of mTOR signaling by rapamycin at a concentration that efficiently inhibited MLR in the same combination, did not influence resistance to ABT-737 (Fig. 21A). An important role of the TCR-calcineurin-NFAT (signal 1) cascade was further confirmed by using the alternative calcineurin inhibitor tacrolimus and the cell permeable NFAT-inhibitor VIVIT-R (Aramburu, Yaffe et al. 1999). The blockade of this pathway at any level increased the percentage of apoptotic cells in allogeneic, but not in syngeneic cultures (data not shown), and it prevented resistance to ABT-737 (Fig. 21B), excluding an off-target effect of CsA and indicating a crucial role for NFAT in preventing T cell apoptosis in the early phase after antigen recognition. The correlation of these findings with the inhibition of up-regulation of A1 by CsA was confirmed at the mRNA and protein level (Fig. 21C-D). Thus, antigen recognition induced an NFAT-dependent up-regulation of A1 that determined resistance to ABT-737 in allo-antigen activated CD8 T cells, and CsA completely prevented this resistance to ABT-737 in activated cells *in vitro*.

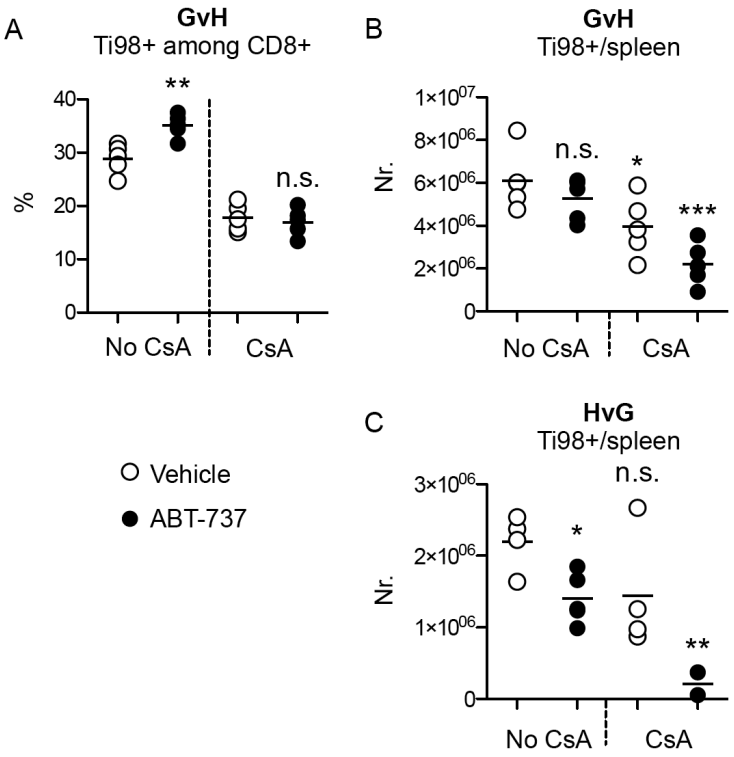


Fig. 22. Cyclosporine A prevents resistance to ABT-737 in vivo

(A-B) (CBAxB6)F1 mice were injected with BM3.3 splenocytes to induce a GvH reaction and treated with ABT-737 (50 mg/kg/day, i.p.) with or without CsA (10 mg/kg/day, s.c.). After 3 days the mice were sacrificed and the spleens analyzed by FACS. (A) Treatment with ABT-737 alone resulted in the selection of allo-reactive Ti98+ cells. However, this phenomenon was completely prevented in combination with CsA (statistical comparison to vehicle or CsA alone respectively). (B) As a result, the total number of allo-reactive CD8+Ti98+ cells per spleen was markedly reduced in the combination therapy group, compared to CsA or ABT-737 alone.

(C) Similarly, in an HvG combination using synchimeric mice (s. Fig. 17 for experimental setup), the combination of ABT-737 and CsA strongly reduced the number of donor-reactive Ti98+ cells as measured in spleens at day 5 after injection of B6 splenocytes. Statistical comparison to vehicle group: n.s. $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Reversibility of ABT-737 resistance by cyclosporine A in vivo

The critical role of signal 1 for ABT-737 resistance offers the opportunity to prevent resistance to ABT-737 using clinically well-established calcineurin inhibitors. We tested this option using a combination of CsA and ABT-737 in the GvH and HvG models introduced before. For the GvH experiments BM3.3 splenocytes were transferred to F1 mice under treatment with low doses of CsA (10 mg/kg/day). Similarly to the previous experiment (Fig. 18), ABT-737 (50 mg/kg/day) was administered at day 1 and 2 after cell transfer, and on day 3 mice were sacrificed for spleen FACS analysis. Concurrent with the *in vitro* results, the selection of activated donor reactive CD8+Ti98+ cells observed in mice treated with ABT-737 alone was completely prevented by the addition of CsA (Fig. 22A). Because of the general lymphopenia induced by ABT-737, this resulted in a much more pronounced depletion of alloreactive T cells in the combination group (Fig. 22B). This effect was even more pronounced in the HvG model, where treatment was continued for 5 days after DST and the immunosuppressive effect of CsA simultaneously contributed to the inhibition of the allogeneic immune response (Fig. 22C). Thus, resistance to ABT-737 after antigen recognition was successfully overcome by combination with low doses of CsA.

Discussion

The regulation of the intrinsic apoptosis pathway in lymphocytes assumes a novel relevance after the advent of small-molecule Bcl-2 inhibitors. Here we present evidence that sensitivity to ABT-737 in lymphocytes dynamically changes during the course of immune responses, and activated T cells are transiently resistant to ABT-737 during the first days after antigen recognition because of a signal 1-dependent NFAT-dependent up-regulation of A1. As a result, ABT-737 displayed a unique selectivity profile in its pro-apoptotic potency, depleting naïve lymphocytes, but sparing T cells after antigen-specific activation.

A1 is up-regulated in the first hours after T cell activation and protects activated thymocytes and splenocyes from premature death (Gonzalez, Orlofsky et al. 2003; Verschelde, Walzer et al. 2003). A1-dependent resistance to ABT-737 has been previously reported in lymphoma cells (Del Gaizo Moore, Schlis et al. 2008; Vogler, Butterworth et al. 2009; Yecies, Carlson et al. 2010). Here we show for the first time that physiological up-regulation of A1 after antigen recognition and ABT-737 resistance in normal (non-neoplastic) T cells are linked. More precisely, T cell activation resulted in a 1'000- to 10'000-fold resistance to ABT-737, a finding reminiscent of the results obtained by Vogler et al. in B cell lymphoma cells cultured with CD154-expressing fibroblasts and IL-2 (Vogler, Butterworth et al. 2009). Mechanistic analyses revealed that the TCR-calcineurin pathway controlled A1 up-regulation and that NFAT was the crucial transcription factor in this context (Verschelde, Walzer et al. 2003), thereby mimicking the mechanism regulating A1 in mast cells after IgE receptor stimulation (Ulleras, Karlberg et al. 2008). Interestingly, fundamentally different pathways were involved in resistance to ABT-737 in CLL cells (which was signal 2- and 3-dependent and NFκB-mediated) suggesting a different regulation of A1 in B and T lymphocytes. In the complex system of apoptosis regulation it is possible that additional factors may influence the sensitivity of T cells to Bcl-2 inhibition during the course of the immune response (e.g. Mcl-1 protein stabilization (Wensveen, van Gisbergen et al. 2010)). However, we demonstrated that blocking A1 was critical to prevent resistance to ABT-737, and inhibiting signal 1 of T cell activation achieves this goal.

The synergistic effect of CsA and ABT-737 is partially paradoxical because of the anti-apoptotic properties of CsA, which have been previously related to a stabilization of the mitochondrial membrane (Zamzami, Marchetti et al. 1996; Hotchkiss, Strasser et al. 2009). This effect is presumably of limited relevance in combination with ABT-737. In contrast, the inhibition of the A1-dependent strong anti-apoptotic signal provided by the Ca^{2+}-calcineurin-NFAT pathway was crucial to prevent resistance to ABT-737 in activated T cells, as shown by the similar effect obtained with tacrolimus and VIVIT-R. Interestingly, a dysregulation of the Ca^{2+}-calcineurin-NFAT pathway has been described in several lymphatic and solid tumors and may therefore influence the anti-neoplastic effect of Bcl-2 inhibitors (Muller and Rao 2010). The most important consequence of the signal 1 dependency of ABT-737 resistance described here is the possibility to easily prevent it by well-established drugs such as calcineurin inhibitors. Characterization of ABT-737 resistance in physiological processes is relevant to find pharmacological strategies to potentiate the effect of Bcl-2 inhibitors, and, although the use of immunosuppressive drugs to control cancer may be counterintuitive and potentially dangerous, we speculate that the combination of calcineurin inhibitors with ABT-737 might be beneficial in selected cases.

Because of the different molecular affinities of small-molecule Bcl-2 inhibitors to different members of the Bcl-2 family (Vogler, Dinsdale et al. 2009), their immunomodulatory effect strictly depends on the expression of different members of the Bcl-2 family in distinct lymphocytes subpopulations and during the different phases of an immune response. Myeloid cells express high levels of Mcl-1 and are not affected by

ABT-737; in contrast, ABT-737 efficiently induces apoptosis in naive lymphocytes (Cippa, Kraus et al. 2011). Here we demonstrate that the physiological mechanisms that protect T cells from apoptosis in the initial phase after antigen recognition dramatically influence their sensitivity to ABT-737. Particularly, the low affinity of ABT-737 to A1 determines a selection of antigen-specific T cells in the first days after activation. Thus, ABT-737 is not effective as an immunosuppressive agent during the first days after transplantation and in the acute phase of an autoimmune disease (Lawlor, Smith et al. 2011), but may find a clinical application for induction therapy before solid organ or stem cell transplantation. In contrast, we speculate that an opposite result would be obtained with a selective A1 inhibitor, but none of the currently available Bcl-2 inhibitors selectively binds to A1. However, the fact that ABT-737 in combination with CsA efficiently depleted activated T cells in a GvH and an HvG model explains the synergistic effect of ABT-737 and CsA, that we previously observed in a skin graft model (Cippa, Kraus et al. 2011), and is a reasonable option to potentiate the immunosuppressive effect of Bcl-2 inhibitors.

Finally, the unique selectivity profile of ABT-737 may find a useful application for cell-based immunotherapy. The experimental selection of antigen-specific cells after a short activation time is difficult to achieve and largely limited to the use of transgenic systems. ABT-737 allows selecting polyclonal antigen-specific cells after antigen recognition *in vitro* and *in vivo* with a wide experimental application in the field of infection and cancer immunology, i.e. for the generation of virus- or tumor-antigen specific T cells presented by the host MHC. As resistance to ABT-737 depends only on signal 1 activation, antigen-specific T cells can be further influenced by interleukins to generate particular subsets of T cells *in vitro*, such as donor-reactive regulatory T cells or CMV-reactive cytotoxic T cells (Sagoo, Ali et al. 2011).

Thus, in this study we first described, characterized and found a way to overcome resistance to ABT-737 in activated T lymphocytes. Moreover, we propose a link between the well-established resistance to ABT-737 in tumor cells and physiological lymphocyte activation after antigen recognition. These findings are relevant for a potential clinical application of Bcl-2 inhibitors as immunomodulatory and anti-neoplastic agents.

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Conflicts of Interest

P.D.B. is an employee of Abbott, which developed and provided ABT-737. However, no financial sponsoring was received for this study, and no conflict of interest exists for the other authors.

Chapter 5: Targeting the apoptosis pathway to induce mixed chimerism and allograft tolerance without myelosuppressive conditioning

Pietro E. Cippà[1,2], Jin Chen[1,2], Philip D. Bardwell[3], Andrew Bushell[4], Annick Guimezanes[5], Martin Hausmann[6], Anna K. Kraus[1,2], Thomas Wekerle[7], Rudolf P. Wüthrich[1,2], Thomas Fehr[1,2]

[1] Institute of Physiology, University of Zürich, Switzerland
[2] Division of Nephrology, University Hospital Zürich, Switzerland
[3] Abbott Bioresearch Center, Worcester, USA
[4] Nuffield Department of Surgical Sciences, University of Oxford, John Radcliffe Hospital, Oxford, UK
[5] Centre d'Immunologie de Marseille-Luminy, Université de la Méditerranée, Marseille, France
[6] Division of Gastroenterology and Hepatology, University Hospital Zürich, Switzerland
[7] Division of Transplantation, Medical University of Vienna, Vienna, Austria

Abstract
Deletion of donor-reactive T cells is the most reliable mechanism to induce allograft tolerance. Apoptosis determines central and peripheral lymphocyte selection and is required to induce deletional tolerance through costimulation blockade. Here, we used a small molecule Bcl-2 inhibitor (ABT-737) to induce mixed chimerism through a direct pharmacological modulation of the intrinsic apoptosis pathway in peripheral T cells. Using a short conditioning therapy with ABT-737 in combination with costimulation blockade (anti-CD154) and low-dose cyclosporine A we established a novel protocol to induce robust systemic deletional tolerance with long-term acceptance of secondary donor-type allografts without any myelosuppressive conditioning. ABT-737 reversed the well-known anti-tolerogenic effect of calcineurin inhibitors in costimulation-blockade-dependent protocols by boosting the role of the pro-apoptotic factor Bim, which was identified here as a critical factor for mixed chimerism induction, and whose expression was dysregulated by cyclosporine A. Thus, tolerance across full MHC can be achieved targeting the apoptosis pathway, without myelosuppressive condition, without risk of graft-versus-host disease and using moderate doses of bone marrow cells. This low toxicity regimen represents a promising option for a potential clinical application.

Introduction

Induction of operational allograft tolerance, a state in which the immune system accepts donor organs without any immunosuppression but normally responds to foreign antigens, represents the ideal solution for chronic graft rejection and immunosuppression-related toxicity after solid organ transplantation (Pascual, Theruvath et al. 2002; Fehr and Sykes 2008). Among the different ways to induce tolerance in rodents, very few were successful in large animal models and only one strategy – namely the induction of mixed chimerism by a combined transplantation of solid organ and hematopoietic stem cells from the same donor – was successful in clinical pilot studies (Fudaba, Spitzer et al. 2006; Kawai, Cosimi et al. 2008; Scandling, Busque et al. 2011). However, the high toxicity of currently available conditioning protocols to induce mixed chimerism in humans has precluded a broad clinical application (Pilat and Wekerle 2010). Recent discoveries on the mechanisms involved in tolerance induction might lead to more selective and less toxic protocols for translation of this concept to the clinic.

Deletion of allo-reactive T cells is the fundamental mechanism of tolerance in mixed chimeras (Sykes 2001; Wekerle, Kurtz et al. 2002). After engraftment of donor-derived cells in the bone marrow (BM) and in the thymus, tolerance is maintained by central deletion of nascent donor-reactive lymphocytes (Wekerle, Sayegh et al. 1998). However, to prevent rejection in the initial phase after transplantation, therapeutic interventions targeting pre-existing allo-reactive T cells in the periphery and in the thymus are required (Fehr, Takeuchi et al. 2005; Fehr, Lucas et al. 2010). This can be achieved by a profound generalized lymphopenia (Sykes, Szot et al. 1997) or by a more selective tolerization of allo-reactive T cells through costimulation blockade combined with alloantigen (Wekerle, Kurtz et al. 2000; Seung, Mordes et al. 2003). Blocking CD28/CD80/CD86 and CD154/CD40 signaling was sufficient to induce tolerance in several mouse models (Larsen, Elwood et al. 1996; Seung, Mordes et al. 2003), but the efficacy of this approach was reduced in case of a high T cell precursor frequency (Ford, Wagener et al. 2008). Therefore, cytotoxic therapies aiming at a general T cell clone size reduction are usually included in conditioning protocols, resulting in higher toxicity and limited clinical applicability.

Tolerance by costimulation blockade is the result of anergy, regulation and deletion of allo-reactive T cells (Wekerle, Kurtz et al. 2002). After BMT under the effect of anti-CD154, selective deletion of alloreactive CD4 and CD8 T cells occurs by activation of the apoptosis cascade (Li, Li et al. 1999). This process is regulated by two interconnected pathways: the extrinsic apoptosis pathway is mediated by cell surface receptors, whereas the intrinsic (or mitochondrial) apoptosis pathway by pro- and anti-apoptotic factors of the Bcl-2 family (Marsden and Strasser 2003). Resistance to anti-CD154 or CTLA4Ig induced tolerance in mice over-expressing the anti-apoptotic factor Bcl-xL in a heart transplantation model (Wells, Li et al. 1999) indicates that the regulation of the intrinsic pathway is pivotal in this setting. The recent advent of selective Bcl-2 inhibitors offers new options to pharmacologically modulate these processes. Of particular clinical interest are the small-molecules ABT-737 and ABT-263 (navitoclax)(Oltersdorf, Elmore et al. 2005), rationally designed molecules with antineoplastic (Vogler, Dinsdale et al. 2009) and immunomodulatory properties (Bardwell, Gu et al. 2009; Carrington, Vikstrom et al. 2010; Cippa, Kraus et al. 2011). ABT-737 acts as a "sensitizer BH3-only protein": it inhibits the anti-apoptotic Bcl-2 factors Bcl-2, Bcl-xL and Bcl-w and enhances the effect of pro-apoptotic endogenous "activator BH3-only proteins", such as Bid or Bim (Oltersdorf, Elmore et al. 2005). Importantly, ABT-737 and ABT-263 were selective for lymphocytes and platelets (Cippa, Kraus et al. 2011), and did not induce myelosuppression, resulting in a favorable toxicity profile in first clinical trials (Wilson, O'Connor et al. 2010).

In this study we investigated the potential role of Bcl-2 inhibitors in combination with costimulation blockade to induce mixed chimerism. The BH3-mimetic ABT-737 displayed a marked tolerogenic effect and reversed the previously described anti-tolerogenic effect of calcineurin inhibitors. Thus, we identified a new pharmacological target to induce tolerance using a low toxicity approach that may find a clinical application in the near future.

Materials and Methods

Mice. C57BL/6 (B6, H-2^b), CBA (H-2^k), BALB/c (H-2^d), BM3.3 (CBA, H-2^k) and Bim knock out mice (Bim-/-, H-2^b) were housed in specific pathogen-free conditions at the University of Zürich. The BM3.3 mouse (Auphan, Curnow et al. 1994), which expresses on all CD8 T cells a transgenic T cell receptor (TCR) selective for a naturally processed octapeptide bound to the allogeneic MHC class I molecule H-2K^b, was kindly provided by A.-M. Schmitt-Verhulst (Guimezanes, Barrett-Wilt et al. 2001). Bim-/- mice were kindly provided by Andreas Strasser (Bouillet, Metcalf et al. 1999). All animal experiments were performed according to protocols approved by the legal authority (Vet-

erinary Office, Canton of Zürich, Switzerland).

Conditioning and BM procedures. Different conditioning protocols were tested as indicated. In general, B6 recipient mice received 1.5 or 3 Gy total body irradiation (TBI) from a ^{137}Cesium irradiator on day -1 with respect to BMT. Hamster anti-mouse CD154 (MR1, 2 mg, purchased from Bio-X-cell, West Lebanon, USA) was administered i.p. 6-12 h before transplantation with 25 x 10^6 (50 x 10^6 where indicated) fully MHC-mismatched CBA BM-cells by tail vein injection. In some experiments, CTLA4Ig (abatacept, 0.5 mg, provided by Bristol-Myers Squibb) was administered at day 2 after BMT. ABT-737 was provided by Abbott Bioresearch (Worcester, USA), was dissolved in polyethylene glycol, Tween 80, dextrose solution and DMSO and injected intra-peritoneally (i.p.) at 50 mg/kg. CsA (Sigma-Aldrich, Buchs, CH) was dissolved in ethanol and cremaphor EL (Sigma-Aldrich), then diluted in PBS and injected subcutaneously (s.c.) at 10 mg/kg. Both ABT-737 and CsA were administered daily from day -3 to day 12, on day -2 and day -1 two mice received two injections for a total of 100 mg/kg of ABT-737 and 20 mg/kg of CsA, on day 0 ABT-737 and CsA were not administered. To monitor the deletion of donor-reactive CD8 T cells, 20x10^6 syngeneic BM3.3 splenocytes were adoptively transferred to CBA recipients before starting the conditioning protocol with B6 BM cells. The transgenic H-2K^b reactive BM3.3 CD8 T cells were monitored over time in peripheral blood in FACS using the clonotypic antibody Ti98 (Buferne, Luton et al. 1992), kindly provided by A.-M. Schmitt-Verhulst, and stained with a secondary PE rat anti-mouse IgG purchased from Becton Dickinson (Basel, CH).

Flow cytometric analysis of chimerism and detection of allospecific antibodies. FACS analyses were performed with a BD-FACSCanto (Becton Dickinson, Basel, CH). Chimerism was analyzed in white blood cells at different time points after BMT, in spleen and thymus as indicated. Donor-derived cells were identified by FITC-conjugated anti-H-2D^k (Becton Dickinson, Basel, CH). The cells were counterstained with anti-CD4-PE, anti-CD8-APC, anti-B220-PE, anti-CD11b-APC, anti-CD11c-APC antibodies purchased from eBioscience (Frankfurt, D). Background signal measured in a naïve B6 mouse was subtracted to determine the % of donor-derived cells. Depletion of CD25 cells was monitored using an anti-CD25-PE antibody (eBioscience, Frankfurt, D). Allospecific antibodies were measured in indirect FACS: CBA and BALB/c splenocytes were incubated with recipients' serum (1:5 dilution in FACS buffer) and subsequently stained with a secondary anti-mouse FITC-conjugated IgG antibody (eBioscience, Frankfurt, D). Mean fluorescence intensity was determined in FACS gating on CD8 positive cells.

Skin grafting. Mice were shaved and anesthetized with ketamine/xylazine. Full thickness tail skin (about 1 cm^2) from CBA (donor) or BALB/c (3rd party) mice were grafted 3 to 6 weeks after BMT and considered rejected when <10% of the graft remained viable. In some experiments a second skin grafting was performed using the same procedure. In one experiment before the second skin transplantation, regulatory T cells were depleted using the anti-CD25 monoclonal antibody PC61 (0.25 mg for 2 week every second day, purchased from Bio-X-cell, West Lebanon, USA).

Mixed lymphocyte reaction (MLR). MLR were performed in 96 wells plates with responder splenocytes stimulated by splenocytes from donor, 3rd party or syngeneic mice at a final concentration of 4 x10^6 cells/ml in RPMI medium containing 10% fetal bovine serum, penicillin 100 U/ml, streptomycin 100 μg/ml, 2-mercaptoethanol 50 μM. T cell proliferation was measured by incorporation of ^{3}H-thymidine (Perkin Elmer, Waltham, USA) added to the culture on day 4 after stimulation. For the selective analysis of alloreactive CD8 T cells *in vitro*, BM3.3 spenocytes were stimulated with CD8 T cell depleted splenocytes from B6 (allogeneic) or CBA (syngeneic) mice and analyzed in FACS gating on CD8 T cells. After cell permeabilization, the level of Bim in alloreactive CD8 T cells was detected in FACS using a poly-

clonal rabbit antibody detecting total Bim (EL, L and S isoforms) purchased from Cell Signaling (Danvers, USA) and then stained with a FITC anti-rabbit IgG (eBioscience, Frankfurt, D). Splenocytes were sorted by automatic magnetic cell separation using an autoMACS pro separator according to the protocols of Miltenyi Biotec (Bergisch Gladbach, D). For polyclonal stimulation of splenocytes anti-CD3 and anti-CD28 antibodies were used (eBioscience, Frankfurt, D). Cell viability was measured by propidium iodide exclusion in FACS.

Statistics. Student t-test was used to compare values between groups. A p-value <0.05 was considered significant. Graph Pad Prism Software Version 5.0 was used for calculations.

Results

Bcl-2 inhibition allows mixed chimerism induction with reduced conditioning

The effect of Bcl-2 inhibitors on mixed chimerism and tolerance induction was first assessed adding the BH3-mimetic ABT-737 (50 mg/kg/day) to an established minimal conditioning protocol consisting of low-dose

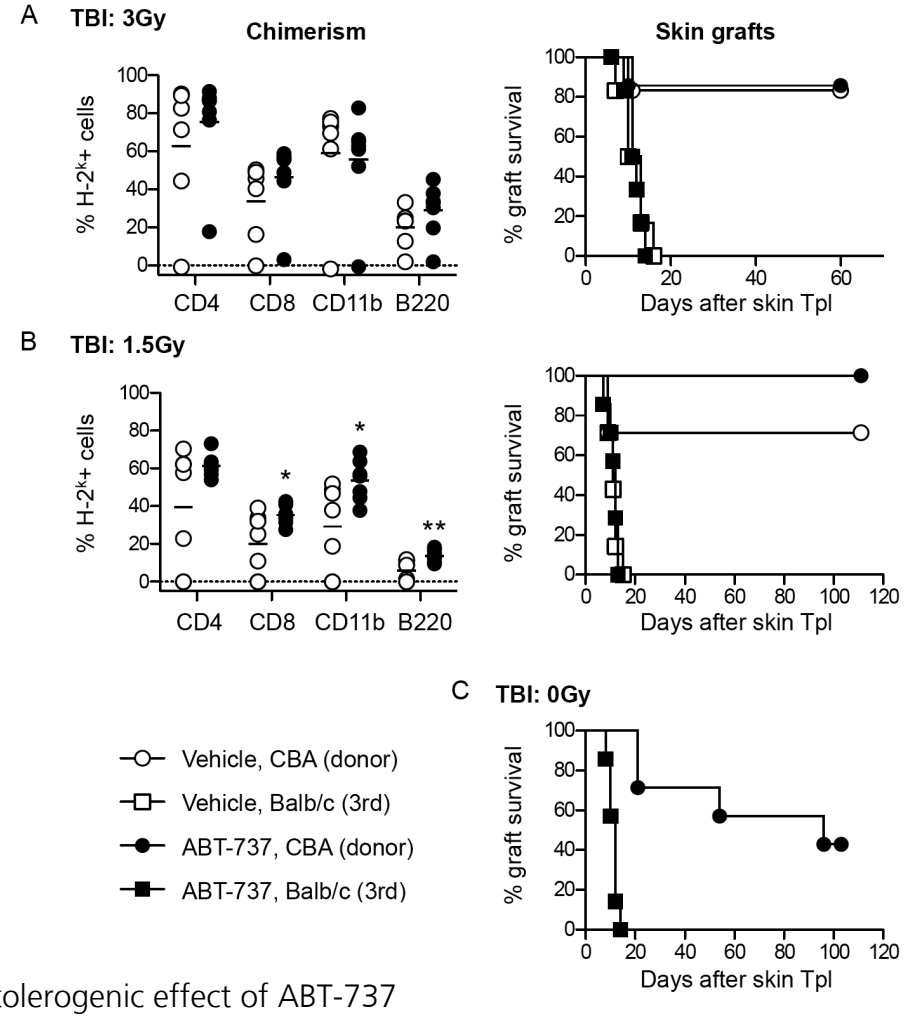

Fig. 23. The tolerogenic effect of ABT-737

ABT-737 was added to a standard conditioning protocol (TBI, MR1, 25 x 10^6 BM-cells). (A) In combination with 3 Gy of TBI similar results were obtained in mice treated with vehicle (white) or ABT-737 (black). Twenty weeks after BMT we measured a similar level of chimerism in both group. After skin transplantation all chimeric mice accepted donor grafts and promptly rejected 3rd party grafts, demonstrating that donor-specific tolerance had been induced. (B) When reducing the dose of TBI to 1.5 Gy, the standard protocol was successful in 5/7 of the recipients. Addition of ABT-737 increased the number of tolerant mice to 8/8 and significantly increased the level of chimerism in blood (week 20 after BMT). (C) In an irradiation free protocol, ABT-737 induced a pronounced donor-specific hyporesponsiveness, but tolerance was not achieved, as shown by the slow rejection of donor skin grafts over time. * p<0.05, **p<0.01.

total body irradiation (TBI, 3 Gy) on the day before BM transplantation, a single injection of anti-CD154 (MR1, 2 mg) and 25x10^6 of fully MHC-mismatched CBA bone marrow cells to B6 recipients. Similar levels of hematopoietic chimerism were obtained in mice treated with ABT-737 or vehicle, and all chimeric animals presented a donor-specific tolerance after skin transplantation (Fig. 23A), indicating that inhibition of Bcl-2, Bcl-xL and Bcl-w was not detrimental for survival and engraftment of donor-derived hematopoietic stem cells (HSCs). To demonstrate a benefit for ABT-737 for mixed chimerism induction, in a second experiment, TBI was progressively reduced to obtain a sub-therapeutic conditioning protocol. In combination with 1.5 Gy TBI, ABT-737 increased the percentage of chimeric mice and induced a higher level of chimerism in all hematopoietic cell lines. All chimeric animals accepted donor-type skin grafts for more than 120 days and promptly rejected third party grafts (Fig. 23B). Mice treated only with ABT-737, MR1 and BM cells but without TBI did not develop significant chimerism (not shown), but skin transplantation (performed 6 weeks after BMT) revealed that this conditioning protocol had induced a marked and lasting donor-specific

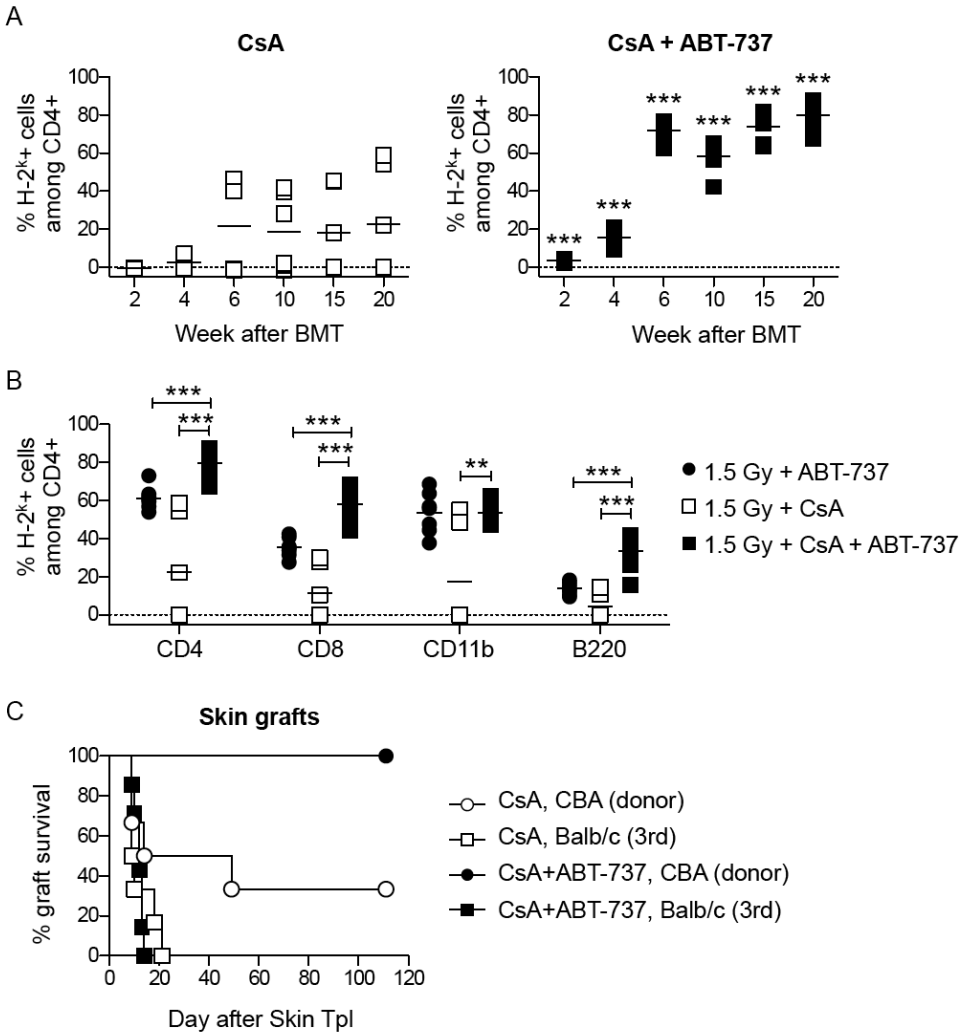

Fig. 24. ABT-737 reverses the anti-tolerogenic effect of CsA

CsA had a deleterious impact on mixed chimerism induction using a standard conditioning protocol (1.5 Gy TBI, MR1, 25 x 10^6 BM-cells). This effect was reversed in combination with ABT-737, as shown in the chimerism kinetics for CD4 T cells (A), in the level of chimerism in different hematopoietic cell lineages 20 weeks after BMT (B) and in the rejection of donor skin grafts (C). Notably in the combination group the chimerism level was even higher than in the group treated with ABT-737 and 1.5 Gy TBI (B). *p<0.05, **p<0.01, ***p<0.001.

hyporesponsiveness (Fig. 23C). Nevertheless, donor grafts were eventually rejected (median survival time 96 days). Thus ABT-737 in combination with CD154 blockade had a tolerogenic effect, but was not sufficient to induce mixed chimerism and stable tolerance in an irradiation-free conditioning protocol.

ABT-737 reverses the anti-tolerogenic effect of calcineurin inhibitors

Reduction of the precursor frequency of donor-reactive T cells is a prerequisite to induce tolerance using costimulation blockade (Ford, Wagener et al. 2008). We hypothesized that a combination with calcineurin inhibitors, which increased the pro-apoptotic potency of ABT-737 on lymphocytes (Cippà *et al*, submitted), might increase the tolerogenic effect of ABT-737. On the other hand, signal 1 inhibition by itself had an anti-tolerogenic effect in costimulation blockade-dependent tolerance induction protocols and interfered with clonal deletion of donor-reactive CD8 T cells (Li, Li et al. 1999; Fehr, Lucas et al. 2010). The above mentioned combination therapy was tested experimentally by adding a short course of low dose CsA (10 mg/kg/day s.c.) to a mixed chimerism protocol using reduced conditioning of 1.5 Gy TBI, MR1, 25x10^6 BM cells with or without ABT-737. According to previous observations (Blaha, Bigenzahn et al. 2003; Fehr, Lucas et al. 2010), treatment with CsA alone resulted in a reduced number of tolerant mice and lower chimerism levels. However, the combination with ABT-737 completely prevented this phenomenon and induced a high level of chimerism and donor-specific tolerance in all recipients. Importantly, the combination of ABT-737 and CsA not only

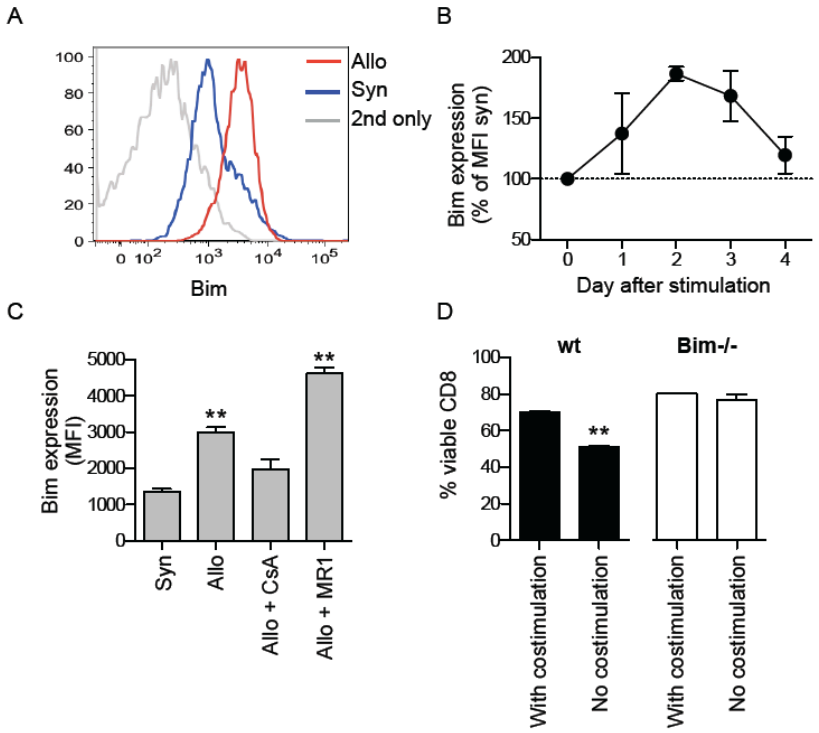

Fig. 25. Regulation of Bim in alloreactive CD8 T cells

(A-B) BM3.3 splenocytes were stimulated with CD8-depleted B6 (allo) or CBA (syn) splenocytes. Allogeneic stimulation induced a transient up-regulation of Bim, with a peak after 2 days of culture. (C) CsA and MR1 substantially influenced the regulation of Bim after antigen recognition: CsA inhibited its initial up-regulation and MR1 prevented its downregulation in the late activation phase. As a result, after 4 days of culture the level of Bim was lower in cells stimulated in the presence of CsA and significantly higher with MR1. (D) Wild type (wt) B6 splenocytes polyclonally stimulated in the presence of MR1 presented a reduced viability of CD8 T cells after 4 days of culture, but the same phenomenon was not observed using Bim-/- cells, suggesting that the reduced down-regulation of Bim determined by MR1 results in cell death in activated CD8 T cells. *p<0.05, **p<0.01.

prevented the anti-tolerogenic effect of calcineurin inhibitors, it even resulted in a higher chimerism level compared to ABT-737 alone, reversing the anti-tolerogenic effect of CsA in a paradoxical synergnism (Fig. 24).

ABT-737 boosts the critical role of Bim in synergism with costimulation blockade

Signal 1 activation leads to an up-regulation of Bim in CD8 T cells (Sandalova, Wei et al. 2004). Therefore, inhibition of this process might explain the anti-tolerogenic of calcineurin inhibitors in anti-CD154 dependent tolerance protocols (Blaha, Bigenzahn et al. 2003; Fehr, Lucas et al. 2010). To further investigate this mechanism, we first established an *in vitro* model to monitor the regulation of Bim in CD8 T cells after alloantigen recognition using a T cell receptor (TCR) transgenic system. BM3.3 CD8 T cells, which express a transgenic TCR specific for the MHC class I molecule H-2K^b (Auphan, Curnow et al. 1994; Guimezanes, Barrett-Wilt et al. 2001), were stimulated with CD8-depleted B6 splenocytes under the effect of CsA or MR1. Expression of Bim was monitored by intracellular FACS over time and correlated with cell viability. Alloantigen stimulation induced a transitory up-regulation of Bim in CD8 T cells (Fig. 25A-B). The initial up-regulation phase was dependent on signal 1 (which can be blocked by CsA), whereas the subsequent down-

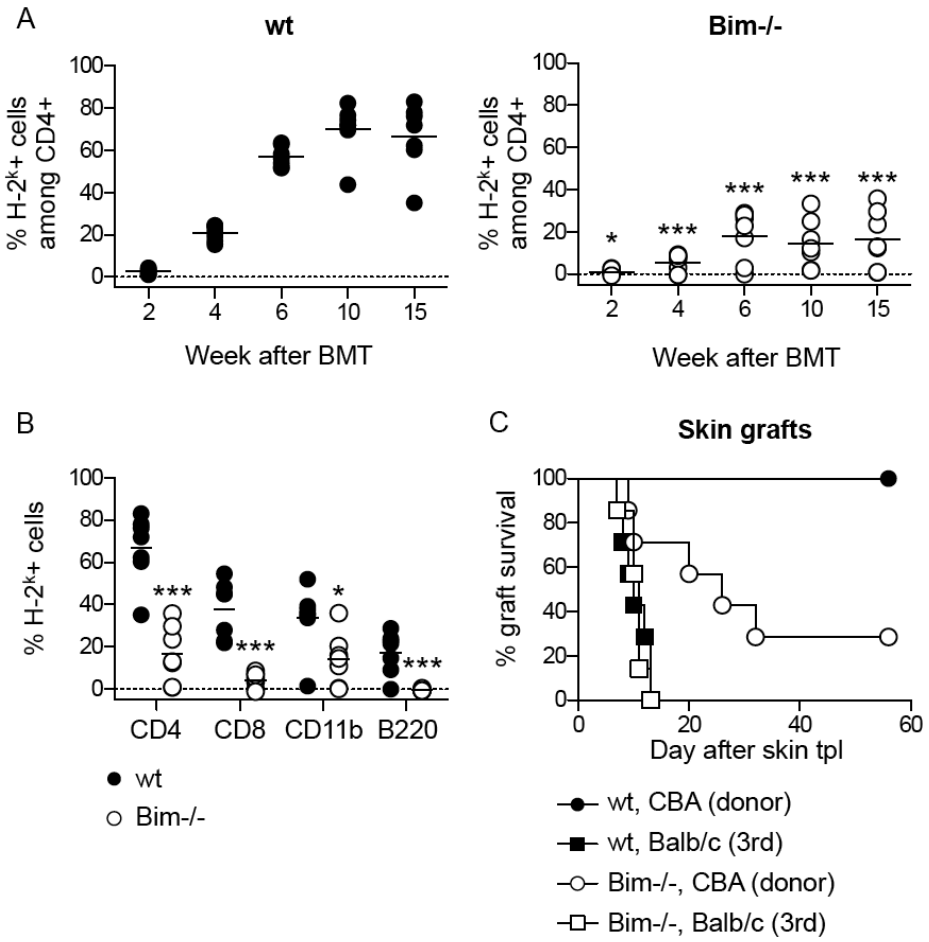

Fig. 26. Resistance to mixed chimerism induction in Bim-/- mice

A standard conditioning protocol (3 Gy TBI, MR1, 25x10^6 BM-cells) induced mixed chimerism in all wild type (wt) B6 mice, but was not successful in most Bim-/- mice, as shown in the chimerism-kinetics for CD4 T cells (A), in the level of chimerism in different hematopoietic cell lineages 15 weeks after BMT (B) and in the rejection of donor skin grafts (C). *p<0.05, **p<0.01, ***p<0.001.

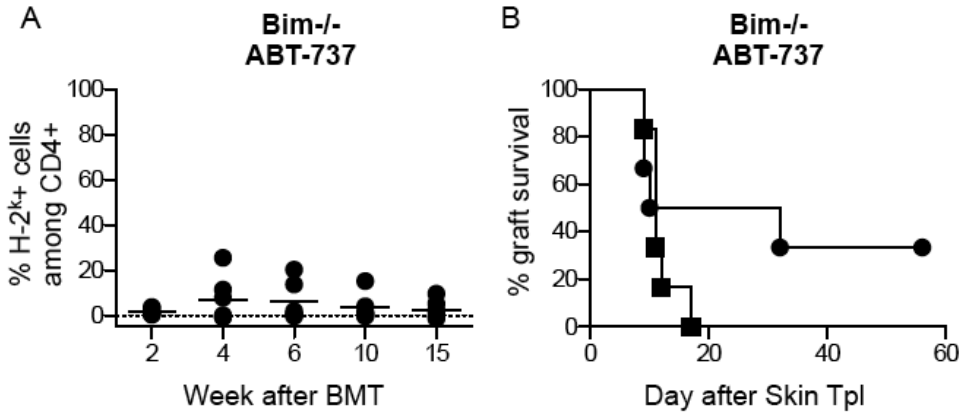

Fig. 27: ABT-737 does not reverse resistance to mixed chimerism induction in Bim-/- mice.
In Bim-/- mice, adding ABT-737 to a standard conditioning protocol (3 Gy TBI, MR1, 25x10^6 BM-cells) we obtained similar results than without ABT-737 (compare with figure 26).

regulation was influenced by costimulatory signals. As a result, the level of Bim remained low in cells activated in the presence of CsA, and – starting at day 3 after stimulation – was significantly higher in CD8 T cells treated with MR1 (Fig. 25C). This correlated with the viability of polyclonally stimulated alloreactive CD8 T cells in vitro: MR1 did not influence alloreactive CD8 T cell viability in culture during the first two days of culture (data not shown), but a progressive loss of viability in alloreactive CD8 T cells stimulated in presence of MR1 was registered at day 3 and 4 after stimulation. This process was completely dependent on Bim, as MR1 did not influence the viability of Bim-/- CD8 in the same experimental conditions (Fig. 25D).
To investigate the role of Bim in the particular situation of mixed chimerism induction, we applied the initially described mixed chimerism conditioning protocol (TBI 3Gy, MR1) to Bim-/- recipients. Compared to wild type animals, Bim-/- mice displayed a marked resistance to mixed chimerism induction, and tolerance was not achieved in most Bim-/- mice (Fig. 26). Thus, in consideration of previous reports about a similar resistance to tolerance induction in transgenic mice over-expressing anti-apoptotic Bcl-xL (Wells, Li et al. 1999), we conclude that the balance between pro- and anti-apoptotic Bcl-2 factors determines deletion of allo-reactive T cells and the anti-tolerogenic effect of CsA in costimulation-blockade depending protocols is indeed related to a dysregulation of the intrinsic apoptosis pathway. More precisely, CsA blocks the initial up-regulation of Bim in alloreactive T cells and prevents a complete deletion of donor-reactive T cells in the late activation phase under the effect of costimulation blockade. In this context, a pharmacological boosting of Bim by ABT-737 might promote the deletion of alloreactive T cells in synergism with costimulation blockade and therefore promote tolerance induction and compensate the anti-tolerogenic effect of CsA. This is confirmed by the fact, that treatment with ABT-737 did not influence the resistance to mixed chimerism induction in Bim-/- mice, further emphasizing the role of Bim in peripheral deletion of allo-reactive T cells as a critical "activator BH3-only protein" in lymphocytes (Fig. 27).

Low toxicity irradiation-free mixed chimerism induction by targeting the apoptosis pathway

The above described synergistic effect of ABT-737 and CsA to induce mixed chimerism was exploited to eventually establish an irradiation-free conditioning protocol. Wild-type B6 mice were treated with ABT-737 (50 mg/kg/day) and low-dose CsA (10 mg/kg/day) for two weeks starting at day -3 with respect to BMT, and an additional dose of both drugs was administered on day -2 in

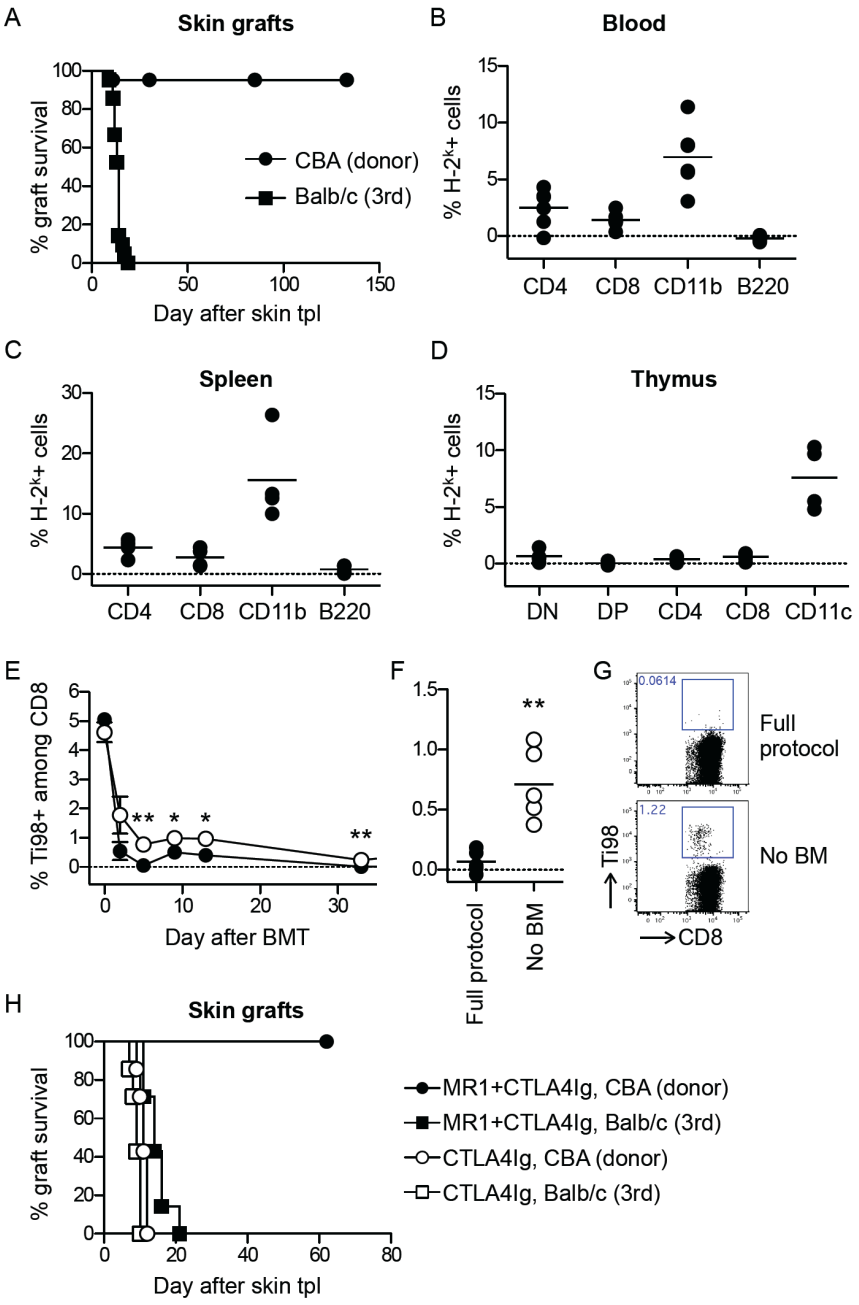

Fig. 28. Irradiation-free mixed chimerism induction with ABT-737 and CsA

An irradiation-free tolerance induction protocol consisting of a two weeks treatment with CsA and ABT-737, MR1 and 25 x 10^6 BM-cells induced donor-specific tolerance. (A) Donor skin grafts were indefinitely accepted by 20/21 mice in 3 independent experiments (max observation time 133 days), and 3rd party grafts were promptly rejected. (B-C) A low level of granulocytes and T cell chimerism was measured in peripheral blood (week 24 after BMT) and in the spleen (33 weeks after BMT). (D) In the thymus double negative (DN), double positive (DP), single-positive CD4 and CD8 lymphocytes did not showed a significant chimerism, but donor derived cells were measured in the CD11c positive fraction.

(E) BM3.3 splenocytes were adoptively transferred to CBA recipients before starting the conditioning protocol with ABT-737, CsA and MR1, 25x10^6 BM cells from B6 donors (full protocol, black dots). In a control group BM cells were not administered (white dots). Donor-reactive BM3.3 CD8 T cells (Ti98+) were reduced in both groups but only in mice receiving the full protocol a complete depletion was achieved: (F-G) after re-challenging with donor antigens (10^6 B6 splenocytes i.v. at day 37) Ti98+ cells were clearly detectable in the control group but had completely disappeared from the peripheral T cell repertoire after exposure to the full conditioning protocol. (H) The conditioning protocol with ABT-737 and CsA was successful in combination with double costimulation blockade with MR1 and CTLA4Ig, but not if MR1 was substituted by CTLA4Ig.

order to achieve a more pronounced peripheral lymphocyte depletion. Subsequently, MR1 was injected 6-12h before $25x10^6$ of BM cells from fully MHC-mismatched CBA donors. This tolerance induction protocol was successful in 20 out of 21 mice in three independent experiments. Donor-type CBA skin grafts were accepted indefinitely with a maximal observation time of >130 days (n=6), whereas all third party grafts (BALB/c) were promptly rejected, therefore demonstrating robust systemic donor-specific tolerance (Fig. 28A). Similarly to previous reports of successful mixed chimerism induction without myelosuppressive conditioning (Wekerle, Kurtz et al. 2000), the level of donor chimerism was rather low compared to protocols including TBI. However this level was stable over time: 33 weeks after BMT we measured a 4.37% of donor-derived CD4, 2.73% CD8 and 15.58% granulocytes in the spleen. Interestingly, the B cell compartment did not show any macrochimerism in the spleen nor in peripheral blood at any time point after BMT (Fig. 28B-C).

Establishment of mixed chimerism with ABT-737, MR1 and CsA leads to robust central and peripheral deletional tolerance

The initial tolerization of peripheral alloreactive T cells was further investigated in an adoptive transfer experiment. Before starting the tolerance induction protocol $20x10^6$ BM3.3 splenocytes were transferred into syngeneic CBA recipients; alloreactive BM3.3 CD8 T cells were monitored in blood over time using the clontypic antibody Ti98. In mice treated with the full conditioning protocol including $25x10^6$ BM-cell from B6 donors, transgenic allo-reactive CD8 T cells completely disappeared from the peripheral T cell repertoire during two weeks and did not recover after resolution of the lymphopenia (Fig. 28E). In contrast, in mice treated with the same pharmacological therapy but without B6 derived BM, Ti98+ cells were markedly reduced but still detectable in peripheral blood. The complete deletion of Ti98+ cells in mice exposed to the full protocol was confirmed by FACS analysis of the spleen after re-challenging the recipient mice with B6 splenocytes (10^6 cells, i.v.) (Fig. 28F-G). Thus, despite the inhibition of signal 1 by CsA, exposure to donor-derived cells was required to induce a complete peripheral deletion of alloreactive T cells.

The success of this conditioning regimen was strictly dependent on inhibition of the CD40/CD154 signal. ABT-737 and CsA alone were not sufficient to induce BM engraftment (data not shown). Adding CTLA4Ig to the conditioning protocol (0.5 mg on day 2 after BMT) resulted in the same positive outcome in terms of donor-specific tolerance and a similar levels of chimerism, whereas CTLA4Ig alone was not sufficient to induce tolerance in this setting (Fig. 28H). By increasing the dose of BM from 25 $x10^6$ to 50 $x10^6$ BM-cells, we obtained a minimal, not significant increase in the chimerism level (data not shown).

Mixed chimeras maintain donor-specific tolerance through central deletion of donor-reactive T cells. The presence of donor-derived antigen presenting cells in the thymus is therefore critical in this setting. Using the above mentioned irradiation-free protocol including ABT-737, we consistently detected about 5% of donor-derived antigen presenting cells (CD11c+) in the thymus (as demonstrated by thymic FACS analysis 33 weeks after BMT; Fig. 28D), a finding consistent with a central clonal deletion mechanism.

The establishment of a sustained systemic tolerance was further confirmed using different immunological tests: donor-specific B cell tolerance was demonstrated by the absence of donor-specific IgG, whereas 3rd party-reactive IgG was readly detected several weeks after skin grafting (Fig. 29A). Non-responsiveness of donor reactive T cells was proven in a classical mixed lymphocyte reaction (Fig. 29B). Finally, 60 days after the first skin transplantation tolerant mice were rechallenged with a second skin graft from donor and 3rd party control. All mice accepted the secondary CBA graft and rejected the BALB/c graft (Fig. 29C-D). The same result was obtained if regulatory T cells were depleted using an anti-CD25 an-

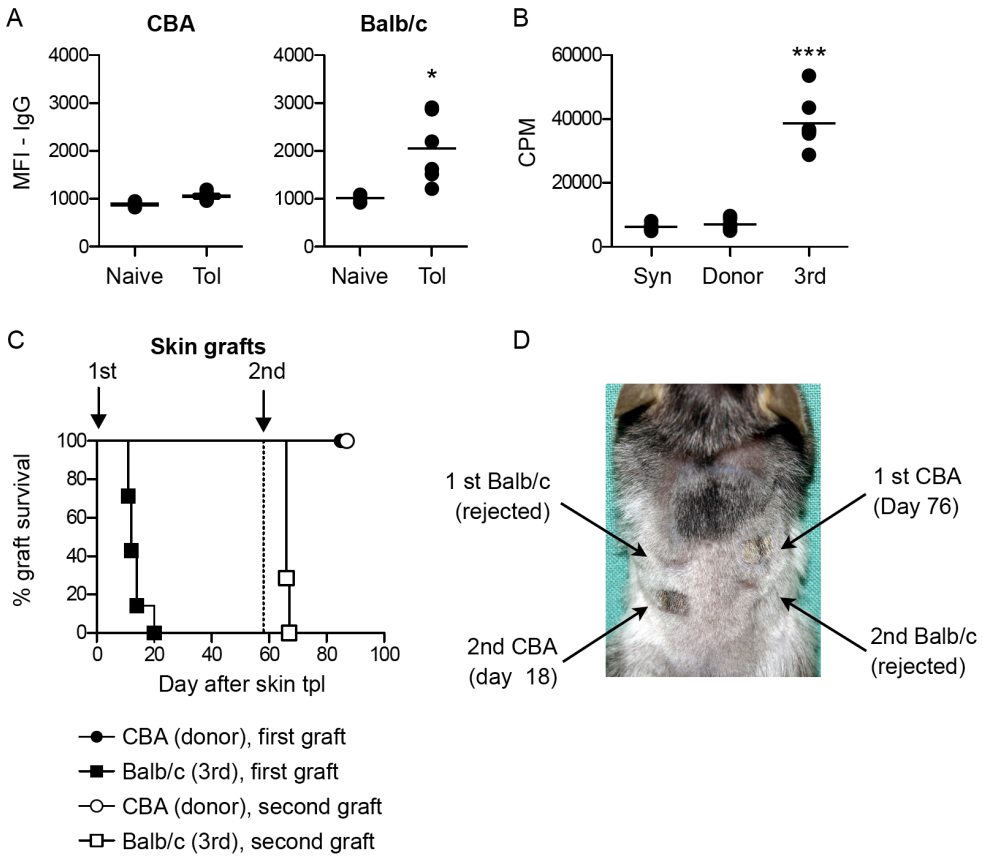

Fig. 29. Robust, systemic tolerance after mixed chimerism induction with ABT-737 and CsA
(A) Sixty days after skin transplantation from CBA and BALB/c donors, mice previously treated with our conditioning protocol (ABT-737, CsA, MR1 and 25 x 10^6 BM-cells) did not present any IgG antibodies reactive with B6 cells, but a normal seroconversion towards BALB/c as measured in indirect FACS. (B) Mixed lymphocyte reaction with splenocytes responders from tolerized mice showed donor-specific non-responsiveness. *p<0.05, **p<0.01, ***p<0.001. (C) A second transplantation at day 58 after first grafting resulted in rapid rejection of 3rd party grafts and acceptance of donor grafts. (D) Picture of a mouse after double skin transplantation. The second two grafts were transplanted, crossed-over, 58 days after the initial transplantation. Both BALB/c grafts were rejected (scar), but the CBA grafts were accepted without signs for rejection. Please note the fur graying (normal phenotype: black), typically observed after combined treatment with CsA and ABT-737.

tibody before re-grafting, indicating that long-term tolerance was independent of classical regulatory T cells (data not shown). The success of this experiment definitively demonstrates that a 2 weeks conditioning protocol with ABT-737 and CsA in combination with MR1 induced mixed chimerism and stable systemic tolerance without myelosuppressive treatment and with clinically relevant doses of BM cells.

Discussion

In this study we identified the intrinsic apoptosis pathway as a novel pharmacological target to induce mixed chimerism and tolerance. More precisely, the balance between pro- and anti-apoptotic factors of the Bcl-2 family was recognized as a critical mechanism for alloreactive CD8 T cell deletion in the context of mixed chimerism and tolerance induction using costimulation blockade. This concept was therapeutically exploited using ABT-737 – a Bcl-2, Bcl-xL and Bcl-w inhibitor – to establish a new irradiation-free protocol to induce robust tolerance with a clinically relevant dose of BM cells.

The regulation of the apoptosis pathway in lymphocytes after antigen recognition is complex. The interaction between T cells and antigen presenting cells influences both branches of the apoptosis pathway and

eventually controls T cell survival (Bouillet and O'Reilly 2009). Focusing on the intrinsic pathway, it has been shown that in the early phase of the immune response signal 1 leads to an up-regulation of the anti-apoptotic Bcl2-A1 that protects the cell from apoptosis without influencing proliferation and induces resistance to ABT-737 ((Verschelde, Walzer et al. 2003), Cippà *et al*, submitted). The initial up-regulation of Bim is counteracted by simultaneous regulation of anti-apoptotic factors and is required for T cell activation (Ludwinski, Sun et al. 2009). Three to four days after antigen-recognition A1 is progressively down-regulated and additional stimuli by costimulatory molecules and interleukins are required to maintain cell survival by a down-regulation of Bim and an up-regulation of Bcl-xL (Sabbagh, Pulle et al. 2008; Watts 2010). In the absence of an adequate anti-apoptotic signal through costimulation, Bim prevails and activated T cells die by apoptosis. This mechanism is crucial to prevent autoimmunity and lymphoproliferative disorders, and – according to previous observations (Li, Li et al. 1999; Wells, Li et al. 1999) and to the data presented here – is critical for deletion of alloreactive T cells in tolerance induction protocols.

Bcl-2 inhibitors offer the opportunity to selectively interact with these mechanisms. In our model ABT-737 promoted peripheral T cell tolerization by at least two complementary effects. First, it boosted the critical role of Bim leading to a more efficient deletion of alloantigen-specific T cells. Second, ABT-737 reduced the precursor frequency by a peripheral deletion of lymphocytes, but avoiding any myelosuppressive effect. Additional effects of Bcl-2 inhibitors on regulatory T cells or directly on BM or thymic engraftment cannot be excluded. The beneficial effect of the combination of ABT-737 and CsA is multi-factorial. CsA potentiated the pro-apoptotic effect of ABT-737 on lymphocytes by a synergistic inhibition of Bcl-2, the crucial survival factor in mature naïve lymphocytes (Veis, Sorenson et al. 1993). Moreover, CsA blocked the up-regulation of A1 preventing resistance to ABT-737 in activated T cells (Cippà *et al*, submitted). On the other hand ABT-737 reversed the anti-tolerogenic effect of CsA by compensating the missing up-regulation of Bim after allostimulation under calcineurin blockade. As a result, the combination of CsA and ABT-737 resulted in a paradoxical synergistic effect and was sufficient to induce tolerance together with MR1 and clinically relevant doses of BM-cells, but without any myelosuppressive treatment.

Apart from the peculiarity of this model to investigate the role of the intrinsic apoptosis pathway in mixed chimerism, this finding is of clinical relevance. As graft loss due to acute rejection cannot be ethically accepted in the modern transplantation era, each clinically applicable tolerance induction protocol has to foresee a combination of initial standard immunosuppressive and tolerance induction regimes in a preventive or therapeutic setting, ideally without *a priori* exclusion of calcineurin inhibitors (Waldmann 2010). Moreover, the option to include CsA to prevent or control GvHD after BMT to induce tolerance, without precluding the efficacy of the tolerance induction regime may be useful in selected clinical cases. Unfortunately, CsA increased the pro-apoptotic potency of ABT-737 not only on lymphocytes: a more pronounced thrombocytopenia and a generalized hair depigmentation were observed in mice treated with ABT-737 and CsA (Cippà *et al*, submitted), but these side effects may be limited when applying a short treatment course only during a tolerance induction protocol.

Our irradiation free protocol induced a low, but stable hematopoietic chimerism at similar level as previously reported in protocols without myelosuppressive conditioning (Wekerle, Kurtz et al. 2000). Because hematopoietic stem cells express high levels of Mcl-1 (Opferman, Iwasaki et al. 2005), ABT-737 does not induce apoptosis in this cell population. Therefore, ABT-737 fails to "create space" in the stem cell niches in the bone marrow, a factor that was originally thought to be required for BM engraftment (Tomita, Sachs et al. 1994). Our data support the hypothesis that depletion of stem

cells is dispensable to induce mixed chimerism. The sustained detection of donor-derived granulocytes over more than 8 months clearly indicates that engraftment of donor stem cells or very early progenitor cells had occurred. However, the level of hematopoietic chimerism was not uniform among different cell lineages with an absence of donor-derived B cells and higher levels of granulocytes. This could be explained by the engraftment of myeloid-biased HSCs (Muller-Sieburg, Cho et al. 2004). This myeloid-biased donor-derived hematopoiesis was sufficient to maintain a pool of donor-derived antigen presenting cells in the thymus to establish central deletional tolerance. In a clinical perspective, this low level of chimerism may be favorable, since it completely avoids the risk of full chimerism induction and graft versus host disease.

The tolerance induction protocol described here provides a solution to several problems currently precluding a broad clinical application of the mixed chimerism approach (Dolgin 2012). The general toxicity of the conditioning regimen is low compared to protocols including myelosuppressive drugs or total lymphoid irradiation (Scandling, Busque et al. 2011). In phase I clinical trials ABT-737 displayed a favorable toxicity profile and its application for a short conditioning therapy seems to be adequate also for non-malignant conditions (Wilson, O'Connor et al. 2010). Depleting antibodies – notably more efficient in mice than in humans (Sachs, Sykes et al. 2011) – are not required, but a blockade of CD40/CD154 was critical in our model. The recent report of antibodies blocking CD40 in advanced pre-clinical studies may represent an ideal solution to block this pathway without the thromboembolic side effect reported in primates after exposure to anti-CD154 (Kawai, Andrews et al. 2000; Page, Srinivasan et al. 2012). Importantly, in contrast to previous reports of mixed chimerism induction without myelosuppression (Wekerle, Kurtz et al. 2000; Pilat, Baranyi et al. 2010), our conditioning protocol was successful using a clinically relevant dose of BM cells and without additional cell-based therapy.

In summary, we describe a novel approach to reliably induce allograft tolerance using the mixed chimerism model and selective targeting of the apoptosis pathway. This approach allowed to establish a non-toxic, non-myelosuppressive conditioning protocol with potential clinical application.

Acknowledgements

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Conflicts of Interest

P.D.B. is an employee of Abbott, which developed and provided ABT-737. However, no financial sponsoring was received for this study, and no conflict of interest exists for the other authors.

Chapter 6: Apoptosis induction in memory T cells to overcome costimulation blockade resistance in transplantation

Pietro E. Cippà[1,2], Jin Chen[1,2], Philip D. Bardwell[3], Thomas Wekerle[4], Annick Guimezanes[5], Rudolf P. Wüthrich[1,2], Thomas Fehr[1,2]

[1] Institute of Physiology, University of Zürich, Switzerland
[2] Division of Nephrology, University Hospital Zürich, Switzerland
[3] Abbott Bioresearch Center, Worcester, USA
[4] Division of Transplantation, Medical University of Vienna, Vienna, Austria
[5] Centre d'Immunologie de Marseille-Luminy, Université de la Méditerranée, Marseille, France

Abstract
Memory T cells are recognized as a major barrier for costimulation blockade and tolerance induction, but currently available therapeutic options have a limited impact on their survival or immunological activity. The intrinsic apoptosis pathway regulates memory T cell survival through the interaction of the pro-apoptotic factor Bim and the anti-apoptotic factors Bcl-2 and Bcl-xL. In this study we investigated the effect the Bcl-2 inhibitor ABT-737 on alloreactive memory T cells. ABT-737 induced apoptosis in memory T cells at very low concentrations *in vitro* and prolonged skin graft survival to the level of a primary response in sensitized mice in a fully MHC-mismatched combination. Moreover, a short induction therapy with ABT-737 in combination with donor-specific transfusion and costimulation blockade with anti-CD154 overcame resistance to costimulation blockade determined by adoptive transfer of memory T cells in naïve mice in a skin graft model and promoted the establishment of donor-specific hyporesponsiveness. Thus, apoptosis induction in memory T cells by Bcl-2 inhibition is a promising approach for immunosuppression and induction of tolerance in sensitized recipients and recipients displaying heterologous immunity such as non-human primates and patients.

Introduction

Costimulation blockade is a promising approach to prevent allograft rejection and induce donor specific tolerance (Ford and Larsen 2009). Blockade of CD28/CD80/CD86 and CD40/CD154 signaling prolonged survival of skin and vascularized allografts (Larsen, Elwood et al. 1996) and allowed to markedly reduce the toxicity of mixed chimerism conditioning protocols in rodents (Wekerle, Kurtz et al. 2000). However, translation of the same protocols to non-human-primates and humans was particularly challenging, suggesting the presence of a substantial difference between the immune system of laboratory mice and humans in this particular context (Ford and Larsen 2011).

Memory T cells are considered one of the most important barrier to costimulation blockade: the adaptive immune system of laboratory mice, young animals that have barely been exposed to pathogens, mostly consists of naïve lymphocytes, whereas in humans a progressive shift from naïve cells to memory cells occurs during life as a result of previous infections and of a progressive decline in thymus function, a processes generally summarized as immune senescence (McElhaney and Effros 2009). Although memory cells primarily recognize pathogen-related antigens, it has been demonstrated that cross-reactive memory cells can recognize alloantigens in a process called heterologous immunity, and therefore assume a critical relevance not only in donor-

sensitized recipients (Adams, Williams et al. 2003).

Memory T cells are characterized by a lower activation threshold, are less dependent on costimulation and are not efficiently inhibited by regulatory T cells (Lanzavecchia and Sallusto 2000; London, Lodge et al. 2000; Yang, Brook et al. 2007). As a result, the efficacy of costimulation blockers is limited in the context of a secondary immune response. Anti-CD154 dependent experimental tolerance protocols are not effective in sensitized mice or after adoptive transfer of memory T cells (Adams, Williams et al. 2003), and the presence of high numbers of memory cells correlated to resistance to tolerance in primates (Nadazdin, Boskovic et al. 2011). Moreover, it is presumed that the higher incidence of acute rejection episodes in patients on a belatacept-based immunosuppression is the direct consequence of the same phenomenon (Vincenti, Larsen et al. 2005; Vincenti, Dritselis et al. 2011).

Compared to naïve cells, memory T cells are more resistant to immunosuppression and depleting antibodies, so that induction therapy with alemtuzumab or rabbit antithymocyte globulin resulted in the selection and accumulation of functional T cells with a memory-like phenotype (Pearl, Parris et al. 2005). Recent studies demonstrated a favorable inhibition of memory T cells by targeting adhesion molecules such as CD2 (notably, an anti-CD2 antibody was also used in the first successful tolerance induction clinical study (Kawai, Cosimi et al. 2008)) (Weaver, Charafeddine et al. 2009) and LFA-1 (Badell, Russell et al. 2010), but the efficacy and safety of these agents in humans remains to be proven.

Apoptosis controls T cell contraction and generation of memory cells at the end of the immune response (Weant, Michalek et al. 2008). Particularly, the balance between the pro-apoptotic BH3-only protein Bim and the anti-apoptotic Bcl-2 and Bcl-xL controls long-term memory T cell survival (Schluns and Lefrancois 2003; Sabbagh, Srokowski et al. 2006; Wojciechowski, Tripathi et al. 2007) and pharmacological Bcl-2 inhibition induced memory T cell depletion in naïve mice (Wojciechowski, Tripathi et al. 2007; Carrington, Vikstrom et al. 2010). ABT-737 and ABT-263 (navitoclax) are small molecule Bcl-2 inhibitors, which bind to anti-apoptotic Bcl-2 family factors Bcl-2, Bcl-xL and Bcl-w and indirectly activate the apoptotic cascade (Oltersdorf, Elmore et al. 2005). ABT-737 showed a marked antineoplastic effect (Khaw, Huang et al. 2011), inhibited autoimmunity and allograft rejection in mouse models (Bardwell, Gu et al. 2009; Cippa, Kraus et al. 2011). Interestingly, the pro-apoptotic effect of these drugs was rather selective for lymphocytes, resulting in a favorable toxicity profile, principally related to lymphopenia and thrombocytopenia in phase I clinical trials with navitoclax (Wilson, O'Connor et al. 2010).

This study was designed to assess the effect of ABT-737 on alloreactive memory T cells. ABT-737 efficiently induced apoptosis in alloreactive memory T cells and prolonged skin graft survival in sensitized recipients. Furthermore, induction therapy with ABT-737 restored sensitivity to costimulation blockade after memory T cell transfer. Thus, depletion of memory cells targeting Bcl-2 overcomes costimulatory resistance in transplantation.

Materials and methods

Mice. C57BL/6 (B6, H-2^{b}), CBA (H-2^{k}), BALB/c (H-2^{d}) and BM3.3 (CBA, H-2^{k}) mice were housed in specific pathogen-free conditions at the University of Zürich. The BM3.3 mouse, which expresses on all CD8 T cells a transgenic T cell receptor (TCR) selective for a naturally processed octapeptide bound to the allogeneic MHC class I molecule H-2K^{b}, was kindly provided by A.-M. Schmitt-Verhulst (Auphan, Curnow et al. 1994; Guimezanes, Barrett-Wilt et al. 2001). All animal experiments were performed according to protocols approved by the legal authority (Veterinary Office, Canton of Zürich, CH).

Fluorescence activated cell sorting (FACS). FACS analyses were performed with a BD-FACSCanto (Becton Dickinson, Basel, CH). Anti-mouse CD3-FITC, CD3-PECy7, CD4-PE, CD8-APC, CD25-FITC, CD25-PE, CD44-FITC,

CD44-PECy7, CD62L-PE and propidium iodide (PI) were purchased from eBioscience (Frankfurt, D). BM3.3 CD8 T cells were detected using the clonotypic antibody Ti98, which selectively binds to the BM3.3 TCR and was kindly provided by A.-M. Schmitt-Verhulst (Buferne, Luton et al. 1992). A secondary PE rat anti-mouse IgG was purchased from Becton Dickinson (Basel, CH).

Generation of alloreactive memory T cells in vitro. BM3.3 splenocytes were stimulated with CD8-depleted B6 splenocytes in tissue-culture flasks in RPMI medium containing, 10% fetal bovine serum, Penicillin 100 U/ml, Streptomycin 100 ug/ml, 2-mercaptoethanol 50 uM. Two days later, CD8 T cells were positively selected by magnetic cell sorting, washed and cultured in fresh tissue-culture flasks in the same medium supplemented with 20 ng/ml rIL-15 (R&D systems Inc., Minneapolis, USA) for additional 5 days and then used for further analyses. All cell separations were performed by automatic magnetic cell sorting using an autoMACS pro separator according to the protocols of MiltenyiBiotec (BergischGladbach, D).

Generation of alloreactive memory cells in vivo. Six to 8 weeks old CBA mice were adoptively transferred with 10^7 syngeneic BM3.3 splenocytes to monitor priming in blood over time. Two days later 10^7 allogeneic B6 splenocytes were injected i.v. for priming. After 5-8 weeks Ti98+ cells displayed the typical phenotype of central memory cells and were used for further investigations *in vitro* or – after no-touch magnetic T cell sorting – transferred to other naïve CBA mice.

ABT-737 sensitivity in vitro. Splenocytes or *in vitro* generated memory cells were cultured in 96-wells plates in medium (s. above) at a final concentration of $4x10^6$ cells/ml in the presence of different concentrations of ABT-737 or vehicle. After 12h of culture cell viability was assessed by PI exclusion in FACS and % of values measured in corresponding vehicle exposed cultures was calculated. ABT-737 was provided by Abbott Bioresearch (Worcester, USA): for *in vitro* experiments ABT-737 was dissolved in DMSO at a concentration of 5 mM and then diluted in culture medium.

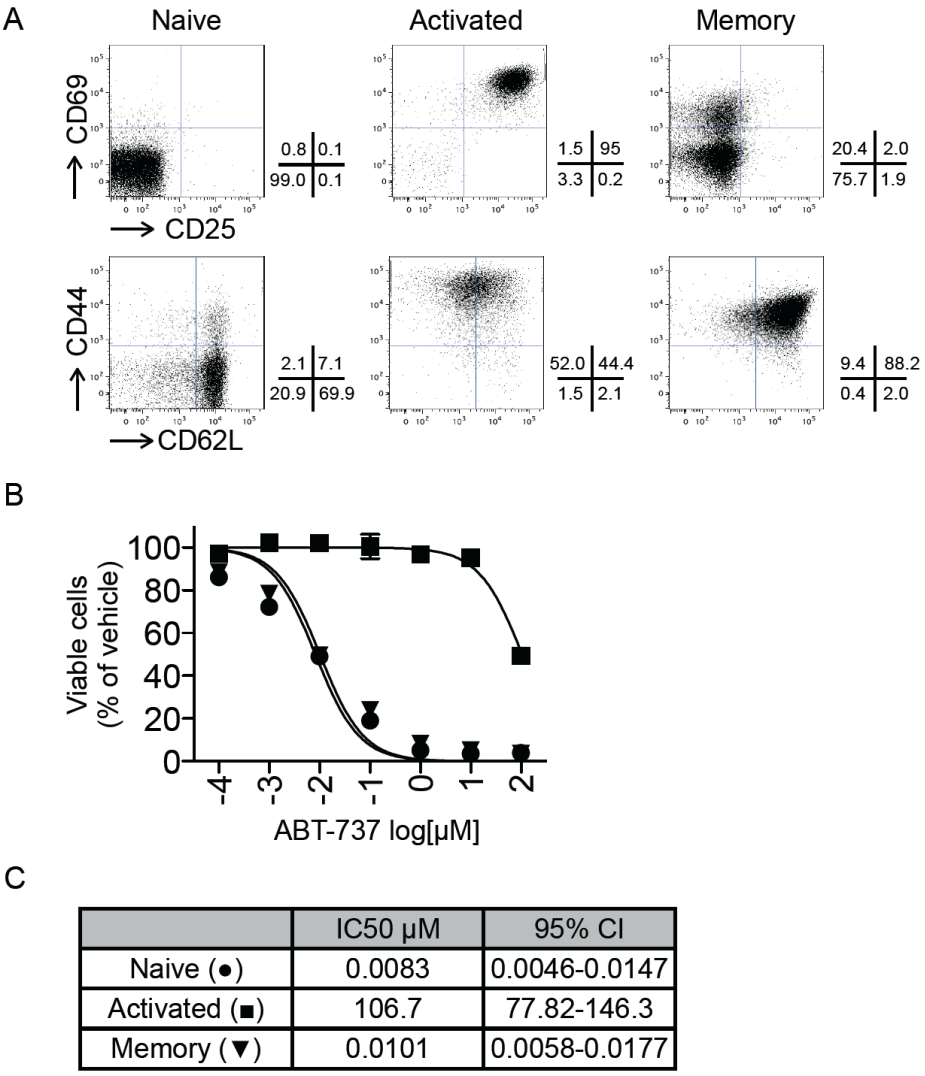
Skin grafting, donor specific transfusion and in vivo treatments. For skin transplantation mice were shaved and anesthetized with ketamine/xylazine. Full thickness tail skin (about 1 cm^2) was grafted and considered rejected when <10% of the graft remained viable. DST was performed by i.v. injection of 10^7 splenocytes. Hamster anti-mouse CD154 (MR1, purchased from Bio-X-cell, West Lebanon, USA) was administered i.p. as indicated. ABT-737 was provided by Abbott Bioresearch (Worcester, USA), was dissolved in polyethylene glycol, Tween 80, dextrose solution and DMSO and injected i.p. at 50 mg/kg.

Statistics. IC50-values were calculated using a log(inhibitor) vs. response model. Skin graft survival was compared using log-rank test. $P<0.05$ was considered significant. Graph Pad Prism Software Version 5.0 was used for calculations.

Results

Apoptosis induction in allo-reactive memory T cells

The pro-apoptotic effect of ABT-737 on alloreactive memory T cells was assessed in two models. First, allo-specific CD8 T cells were generated *in vitro* using a modified version of a previously reported protocol to generate virus-specific memory cells using TCR-transgenic CD8 T cells (Manjunath, Shankar et al. 2001). In our allo-specific system, we used the BM3.3 mouse, which expresses a transgenic TCR specific for the MHC class I molecule H-$2K^b$ on all CD8 T cells (Auphan, Curnow et al. 1994; Guimezanes, Barrett-Wilt et al. 2001). BM3.3 splenocytes were stimulated with CD8-depleted B6 splenocytes for two days. Thereafter, CD8 T cells were positively selected from the mixed lymphocyte reaction culture by magnetic cell sorting and further cultivated in the presence of IL-15 to generate central memory cells. After 5 days we obtained CD8+ transgenic (Ti98+) cells with the phenotypic characteristics of central memory cells (CD25-CD69-CD44+CD62L+)



	IC50 µM	95% CI
Naive (●)	0.0083	0.0046-0.0147
Activated (■)	106.7	77.82-146.3
Memory (▼)	0.0101	0.0058-0.0177

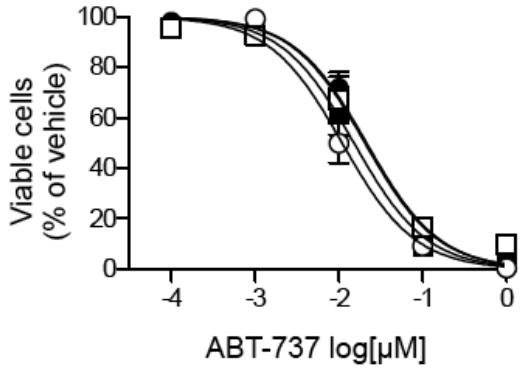
Fig. 30. *In vitro* generated alloreactive memory CD8 T cells are sensitive to ABT-737

BM3.3 splenocytes were stimulated with CD8-depleted B6 splenocytes. Two days later activated CD8 T cells ("activated") were positively selected and further cultured for additional 5 days to obtain memory CD8 T cells ("memory"). (A) Phenotype characterization of naïve, activated and memory T cells by FACS. (B-C) After incubation with different concentrations of ABT-737 or vehicle for 12h cell viability was assessed by PI exclusion in FACS. Naïve and memory cells displayed a similar sensitivity to ABT-737, whereas 1'000-10'000 fold higher concentrations of ABT-737 were required to induce apoptosis in early activated CD8 T cells.

that allowed us to precisely investigate the pro-apoptotic effect of ABT-737 on a homogeneous population of alloreactive memory cells (Fig. 30A). The sensitivity to ABT-737 was assessed by testing viability by propidium iodide exclusion after 12h incubation with different concentration of ABT-737 compared to DMSO-containing vehicle (Fig. 30B-C). *In vitro* generated central memory CD8 T cells were as sensitive to ABT-737 as naïve CD8 T cells and were killed by nanomolar concentrations of ABT-737. In contrast, in accordance to our previous observations (Cippà *et al.*, submitted), CD8 T cells were much more resistant to ABT-737 in the first days after allostimulation, as shown by a 1'000-10'000 higher IC50 measured for cells that were treated with ABT-737 after 2 days of stimulation, but without additional IL-15 incubation (activated cells in Fig. 30).

These results were validated in an *ex vivo* model. After adoptive transfer of BM3.3 splenocytes, CBA mice were primed with transfusion of B6 splenocytes. Eight weeks after priming >80% of Ti98+ cells in the

A



B

	IC50 μM	95% CI
CD8+CD44- (●)	0.022	0.016-0.028
CD8+CD44+ (■)	0.015	0.011-0.021
CD4+CD44- (○)	0.011	0.007-0.015
CD4+CD44+ (□)	0.020	0.014-0.029

Fig. 31. *In vivo* generated alloreactive memory T cells are sensitive to ABT-737
CBA mice were primed with B6 splenocytes. Eight weeks after priming sensitivity to ABT-737 *in vitro* as determined by measurement of cell viability after 12h incubation with ABT-737. Naïve (CD44-) and memory (CD44+) CD4 and CD8 T cells were similarly sensitive to ABT-737. Similar results were obtained as for *in vitro* generated memory cells (Fig. 30).

spleen displayed the typical memory phenotype (CD25-CD69-CD44+), confirming that allospecific priming had occurred (not shown). ABT-737 sensitivity in different T cell populations in these primed mice was then assessed by measuring viability in FACS after 12h of incubation with different concentrations of ABT-737. Naïve (CD44-) and memory (CD44+) CD4 and CD8 T cells displayed a similar sensitivity to ABT-737 (Fig. 31). Thus, *in vitro* and *in vivo* generated memory T cells are sensitive to Bcl-2 inhibition.

Skin transplantation in primed mice

In consideration of the high sensitivity to Bcl-2 inhibition measured in memory T cells, we hypothesized that induction therapy with ABT-737 might prolong skin graft survival in sensitized mice. ABT-737 inhibited allogeneic immune responses in naïve mice, but ABT-737 as a single agent was not sufficient to prolong skin graft survival in a fully MHC-mismatch combination. This was related to the up-regulation of the anti-apoptotic factor Bcl-2A1 in early-activated T cells, which dramatically reduced the pro-apoptotic potency and therefore the immunosuppressive effect of ABT-737 in the first days after antigen recognition (Cippà *et al.*, submitted). However, this phenomenon should not compromise the effect of ABT-737 used for induction therapy. Therefore, in a fully MHC-mismatched skin graft model, ABT-737 was administered to allosensitized mice to reduce the alloreactive memory T cell frequency before re-transplantation. B6 mice were primed with BALB/c skin and 7 weeks later re-transplanted with BALB/c skin under treatment with ABT-737 or vehicle, started 5 days before transplantation. As expected, ABT-737 did not influence skin graft survival in naïve mice. In contrast, ABT-737 prolonged skin graft survival in primed mice, resulting in a similar skin graft survival in primed mice treated with ABT-737 and in naïve mice (Fig. 32). This confirms that depletion of memory T cells by ABT-737 also occurs *in vivo* and is functionally relevant in

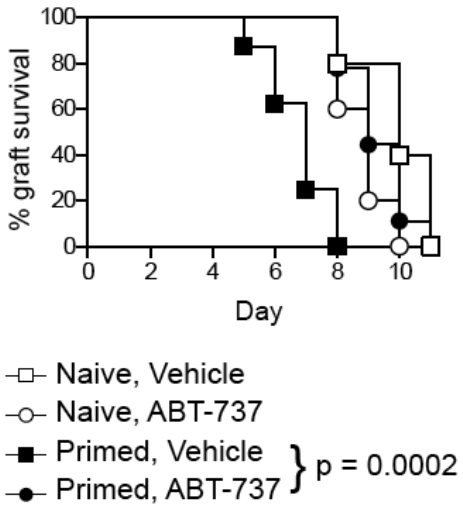


Fig. 32. ABT-737 prolongs skin graft survival in primed mice

Seven weeks after priming with BALB/c skin grafts, B6 recipients were re-transplanted with BALB/c skin on the contra-lateral site. ABT-737 or DMSO-containing vehicle were administered starting on day 5 before transplantation with a daily i.p. injection at 50 mg/kg/day. ABT-737 was not efficient in naïve mice, but significantly prolonged skin graft survival in primed recipients (n=5-9 mice/group).

a strong immunogenic allo-specific model. The kinetics of skin rejection suggests that depletion of memory cells by ABT-737 re-converted a secondary to a primary immune response.

Response to costimulation blockade after transfer of memory cells

The depleting effect of ABT-737 on memory cells may restore sensitivity to costimulation blockade in sensitized recipients. This hypothesis was tested in a fully MHC-mismatched combination using a previously described model including donor specific transfusion (DST), costimulation blockade with anti-CD154 (MR1) and skin transplantation (Markees, Phillips et al. 1997). As antibody removal is not feasible in mice, to obviate the problem of antibody-mediated rejection, an adoptive transfer strategy was chosen. CBA mice were transferred with 10^7 BM3.3 splenocytes and primed with a single transfusion of B6 cells. Priming was confirmed by expression of CD44 in alloreactive Ti98 CD8 T cells (Fig. 33A). After 12 weeks, these mice were euthanized, T cells were isolated from the spleens by no-touch magnetic cell sorting and transferred to naïve CBA recipients ($5x10^6$ cells/mouse). An intensified 2-day induction therapy with ABT-737 was started on the same day. At day 2 after adoptive transfer, DST (B6 splenocytes) and MR1 were administered according to previous reports. MR1 and DST induced a donor-specific hyporesponsiveness with a prolonged mean skin graft survival of 17 days compared to 9 days in the control group (Fig. 33B). However, in analogy to previous observations (Adams, Williams et al. 2003), this effect was lost after transfer of memory T cells. The short induction therapy with ABT-737 dramatically prolonged B6 skin graft survival in mice transferred with naïve T cells and completely prevented resistance to costimulation blockade in mice transferred with memory T cells. Thus, a short induction therapy with ABT-737 greatly amplified the donor-specific hyporesponsiveness induced by DST and MR1.

Discussion

In this study we showed that the Bcl-2 inhibitor ABT-737 induced apoptosis in memory T cells and prolonged skin graft survival in sensitized recipients. Induction therapy with ABT-737 markedly promoted the induction of donor-specific hyporesponsiveness in combination with DST and anti-CD154 and prevented resistance to costimu-

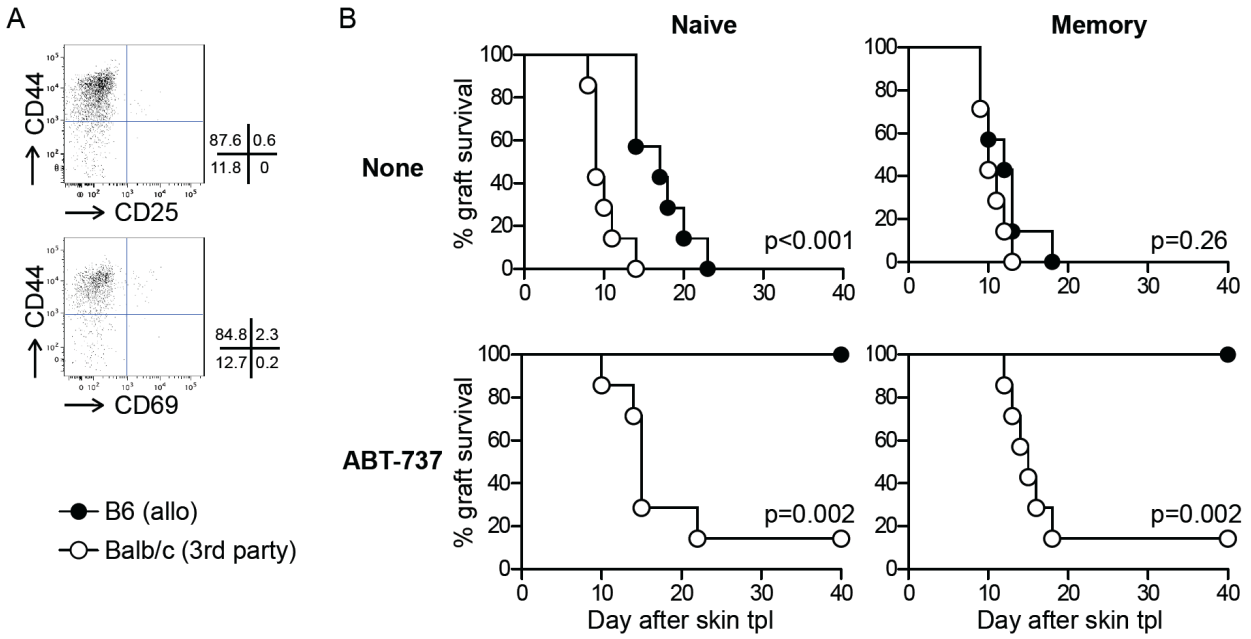


Fig. 33. ABT-737 restores sensitivity to costimulation blockade after transfer of memory T cells
Naïve CBA mice were transferred with syngeneic BM3.3 splenocytes and primed with B6 splenocytes (10^7 cells, i.v. injection). (A) Priming was monitored analyzing the phenotype of alloreactive CD8+Ti98+ T cells in FACS. Twelve weeks after priming Ti98 cells displayed the typical phenotype of memory T cells (CD25-CD44+CD69-). (B) CBA mice were euthanized, T cells isolated from the spleen by negative T cell sorting and transferred to naïve CBA mice (day -9 before skin transplantation). Control mice transferred with naïve T cells were included. Induction therapy with ABT-737 was started at the same day of adoptive transfer and continued at the following day with two injections of ABT-737 (50/mg/kg/injection). At day -7 recipient mice received donor specific transfusion (DST, 10^7 B6 splenocytes). Anti-CD154 (MR1, 0.25 mg/injection, i.p.) was administered at day -7, -3, -1, 3 and 7. Skin transplantation from B6 and BALB/c donors was performed at day 0. Donor-specific hyporesponsiveness induced by DST and MR1 was prevented by adoptive transfer of memory cells, but ABT-737 reversed this phenomenon and induced a much more pronounced hyporesponsiveness in both groups transferred with naïve and memory cells (n=7 mice/group).

lation blockade determined by memory T cells.

The immuno-modulatory effect of Bcl-2 inhibitors is principally related to their selectivity profile among different lymphocyte subpopulations. ABT-737 (and its bioavailable counterpart navitoclax) induces apoptosis in cells whose survival depends on the anti-apoptotic effect of Bcl-2, Bcl-xL or Bcl-w (Oltersdorf, Elmore et al. 2005). Generation and maintenance of memory T cells is the result of a tight regulation of the intrinsic apoptosis pathway. Particularly, down-regulation of Bim is crucial for CD8 memory T cell generation (Sabbagh, Srokowski et al. 2006), and the anti-apoptotic factors Bcl-2 and Bcl-xL are the most important determinants of memory T cell survival (Schluns and Lefrancois 2003; Wojciechowski, Tripathi et al. 2007). By inhibiting both Bcl-2 and Bcl-xL, ABT-737 efficiently induced apoptosis in memory T cells. Previous studies demonstrated that ABT-737 reduced the number of naïve and memory cells in non-sensitized mice *in vivo* (Wojciechowski, Tripathi et al. 2007; Carrington, Vikstrom et al. 2010). Our data complete this information with a quantitative analysis about the sensitivity to ABT-737 in allo-reactive memory T cells *in vitro* and with functional tests *in vivo*. We demonstrate that sensitivity to ABT-737 is similar for naïve and memory cells and that the depleting effect of ABT-737 on memory cells is sufficient to inhibit secondary immune responses.

Apoptosis induction in memory T cells might represent an innovative option to prevent allograft rejection in sensitized patients. Al-

though immunological risk evaluation and therapy of sensitized recipients are mostly focused on B cells and donor specific antibodies (Gloor and Stegall 2010), T cells are surely involved in these mechanisms and therapeutic strategies to inhibit memory T cells are of clinical relevance. Further investigations are required to assess the effect of ABT-737 on B cells and particularly on plama cells and memory B cells to fully exploit this approach. Furthermore, because of the critical role of heterologous immunity, memory T cell depletion is also relevant for the treatment of patients without a detectable donor-specific sensitization in the context of costimulation blockade or immunomodulatory approaches based on regulatory T cells, both notoriously not very efficient in controlling secondary immune responses.

We recently showed that ABT-737 promotes mixed chimerism and tolerance induction in naïve mice (Cippà *et al*, unpublished). The data presented here represent a step toward a broad clinical application of this approach. Memory T cells are more and more recognized as a critical resistance factor to tolerance in primates and humans (Ford and Larsen 2011; Yamada, Boskovic et al. 2011). Previous studies indicate that not only intrinsic characteristics of memory T cells are important in determining resistance to tolerance, but that quantitative aspects may be relevant too (Adams, Williams et al. 2003; Nadazdin, Boskovic et al. 2011). The intrinsic properties of memory cells did not represent a barrier for the pro-apoptotic effect of ABT-737, but we cannot assume that a complete deletion of the alloreactive T cell pool had occurred after a short induction therapy. It seems rather that ABT-737 induced a sufficient clone size reduction in the alloreactive memory T cell pool to restore sensitivity to costimulation blockade. Thus, donor-specific hyporesponsiveness is primarily the result of antigen recognition under the effect of costimulation blockade, but this effect was promoted after clone size reduction by ABT-737 in both situations of a naïve and a primed mouse. Furthermore, an additional direct interaction with the process of alloantigen-specific peripheral lymphocyte selection may also be involved.

This consideration is important also for the potential side effects: if a complete deletion of memory T cells does not occur, a reactivation of infectious diseases as a result of impaired immunological memory seems very unlikely. In consideration of phase I clinical trials with navitoclax, it seems that an induction therapy with Bcl-2 inhibitors should have an acceptable toxicity also in the context of a non-malignant condition.

Thus, apoptosis induction in memory T cells by Bcl-2 inhibition represents a promising strategy to inhibit secondary immune responses and to increase sensitivity to costimulation blockade. This approach might be important for the translation of tolerance induction protocols in models with a high impact of memory cells and toward the clinic.

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Section III: Discussion

Chapter 7: A new class of immunomodulatory drugs

In this study I identified and characterized previously unknown immunomodulatory effects of Bcl-2 inhibitors on allogeneic immune responses. As a single agent, ABT-737 had a limited effect on skin allograft rejection, but a strong immunosuppression was obtained if resistance to ABT-737 in early-activated T cells was prevented by calcineurin inhibition. Moreover, ABT-737 displayed a marked tolerogenic effect in combination with costimulation blockade and allowed us to establish a novel mixed chimerism induction protocol selectively targeting the apoptosis pathway to achieve transplantation tolerance without any myelosuppressive treatment. Finally, the depleting effect of ABT-737 on memory T cells was exploited to overcome resistance to costimulatory blockade in transplantation. Thus, the apoptosis pathway represents a new pharmacological target to prevent allograft rejection.

Why Bcl-2 inhibitors? Why ABT-737?

Because of the fundamental function of apoptosis in animal cells, the first important issue to be considered for the development of therapeutic strategies targeting the apoptosis pathway is the **tissue selectivity** of the candidate drug, which eventually determines its toxicity profile. This aspect is particularly important in the context of transplantation, a non-malignant condition with an alternative therapeutic option. The negative experience of previous attempts to trigger Fas to induce apoptosis in alloreactive T cells, finally abandoned because of major side effects in preclinical studies, is an instructive example in this setting (Owen-Schaub, Yonehara et al. 1992; Ogasawara, Watanabe-Fukunaga et al. 1993).

Among the different approach to activate the apoptosis pathway for the treatment of cancer, the most advanced studies are related to the inhibition of Bcl-2 proteins (s. chapter 2). Moreover, studies in **transgenic mice** showed that deletion of Bcl-2 genes mostly results in a phenotype in the immune system, but that the effect on other organs is quite limited, giving the hope for selectivity for immune cells and an acceptable systemic toxicity after treatment with selective **Bcl-2 inhibitors** (Veis, Sorenson et al. 1993; Bouillet, Metcalf et al. 1999). This was confirmed in animal studies and in the first clinical trials.

The selectivity profile of Bcl-2 inhibitors depends in large part on their binding affinity to different Bcl-2 family proteins correlated with the expression of pro- and anti-apoptotic factors by different cell populations. The most favorable toxicity profile, characterized by lymphopenia and thrombocytopenia, was registered after treatment with **ABT-737** and its bioavailable counterpart **navitoclax** (Wilson, O'Connor et al. 2010). Depletion of lymphocytes is inevitably related to the immunomodulatory effect of ABT-737 (s. below), but thrombocytopenia might represent a limiting side effect in a clinical setting, particularly if treatment with ABT-737 would occur at the time of surgical procedures. Moreover, mice treated with ABT-737 showed a discoloration of the fur, because of a progressive melanocyte loss, that was increased in combination with CsA (Cippa, Kamarachev et al.). Although this side effect should be expected in consideration of the phenotype of Bcl-2 deficient mice (Veis, Sorenson et al. 1993), it has never been reported in patients.

Apart from their favorable toxicity profile, ABT-737 and navitoclax are of particular

clinical relevance because of their selectivity and uniquely **high binding affinity** to Bcl-2 proteins, which may explain their particular efficacy *in vivo* (Oltersdorf, Elmore et al. 2005). Furthermore, preclinical and clinical studies showed a high similarity of the (side) effect profile of navitoclax in mice and patients, confirming that – because these factors are highly **conserved throughout evolution** – results obtained in animal models can probably directly be translated into the clinic (Khaw, Huang et al. 2011).
Thus, although drugs targeting other factors of the apoptosis pathway may provide a good alternative in this setting, the Bcl-2 inhibitors ABT-737 and navitoclax with their high selectivity and acceptable toxicity profile are the most promising drugs in advanced clinical evaluation to modulate the immune system by targeting apoptosis.

The immunomodulatory mechanism of Bcl-2 inhibitors

From our results in an allospecific model we can extrapolate some general considerations about the characteristics of Bcl-2 inhibitors as a novel class of immunomodulatory drugs. As mentioned in the introduction, pro-apoptotic drugs may find a therapeutic application (1) to treat diseases with a primary dysfunction of apoptosis (such a tumors or genetic deficiencies), (2) to eliminate "dangerous cells" or (3) to modulate apoptosis-dependent processes. In our setting the second and the third mechanism are important.
The first immunomodulatory mechanism of action of Bcl-2 inhibitors is related to the **depletion of lymphocytes**. Depletion of harmful immune cells is a fundamental process in the maintenance of self-tolerance and is physiologically mediated by apoptosis. The general idea to deplete lymphocytes to inhibit undesired immune reactions is not new. Depleting **antibodies** have been used for many years in the clinic. Through complement-dependent lysis or phagocytosis stimulation, depleting antibodies lead to a sustained but incomplete lymphopenia, which is associated with an increased risk for infections and lymphomas (Morris and Knechtle 2008). Moreover, memory cells are more resistant to antibody-induced depletion, and recovery after exposure to depleting antibodies is in part mediated by homeostatic proliferation of this cell population (which may be particularly detrimental for the induction of tolerance) (Pearl, Parris et al. 2005). Innovative is however the idea to try to modulate the physiological mechanisms regulating lymphocyte survival – namely the **apoptosis pathway** – to modulate the immune response.
The immunomodulatory outcome of such a depletional strategy inevitably depends on its **selectivity** among different lymphocyte subpopulations. This can be exemplified by two extreme situations: an efficient depletion of allo-reactive CD8 T cells induces tolerance in the mixed chimerism model (Kurtz, Ito et al. 2001), whereas a depletion of Tregs induces autoimmunity (Kim, Rasmussen et al. 2007). We found that the sensitivity to different Bcl-2 inhibitors varies significantly during the course of the immune response. This process is related to a **dynamic regulation** of the intrinsic apoptosis pathway in response to intrinsic and extrinsic stimuli (Fig. 34). Thus, naïve T cells, whose survival depends on Bcl-2, were efficiently depleted by ABT-737, but early activated T cells expressing A1 were completely resistant to the treatment. As a result, the combination of donor specific transfusion and ABT-737 did not induce the desired selective depletion of allo-reactive T cells, but rather their selection among the T cell pool, in a kind of **reversed alloselectivity**, whereas the pro-apoptotic potency of the pan-inhibitor obatoclax was not influenced by T cell activation *in vitro*. To selectively induce apoptosis in early-activated T cells an A1 inhibitor may be appropriate, but none of the currently available Bcl-2 inhibitors displayed these particular binding affinity. The characterization of the molecular mechanisms leading to the A1-dependent resistance to ABT-737 in early-activated T cells allowed us to overcome this problem by inhibiting the calcineurin-NFAT pathway. Notably, because of the general sensitivity of naïve T cells to ABT-737, allo-selectivity can-

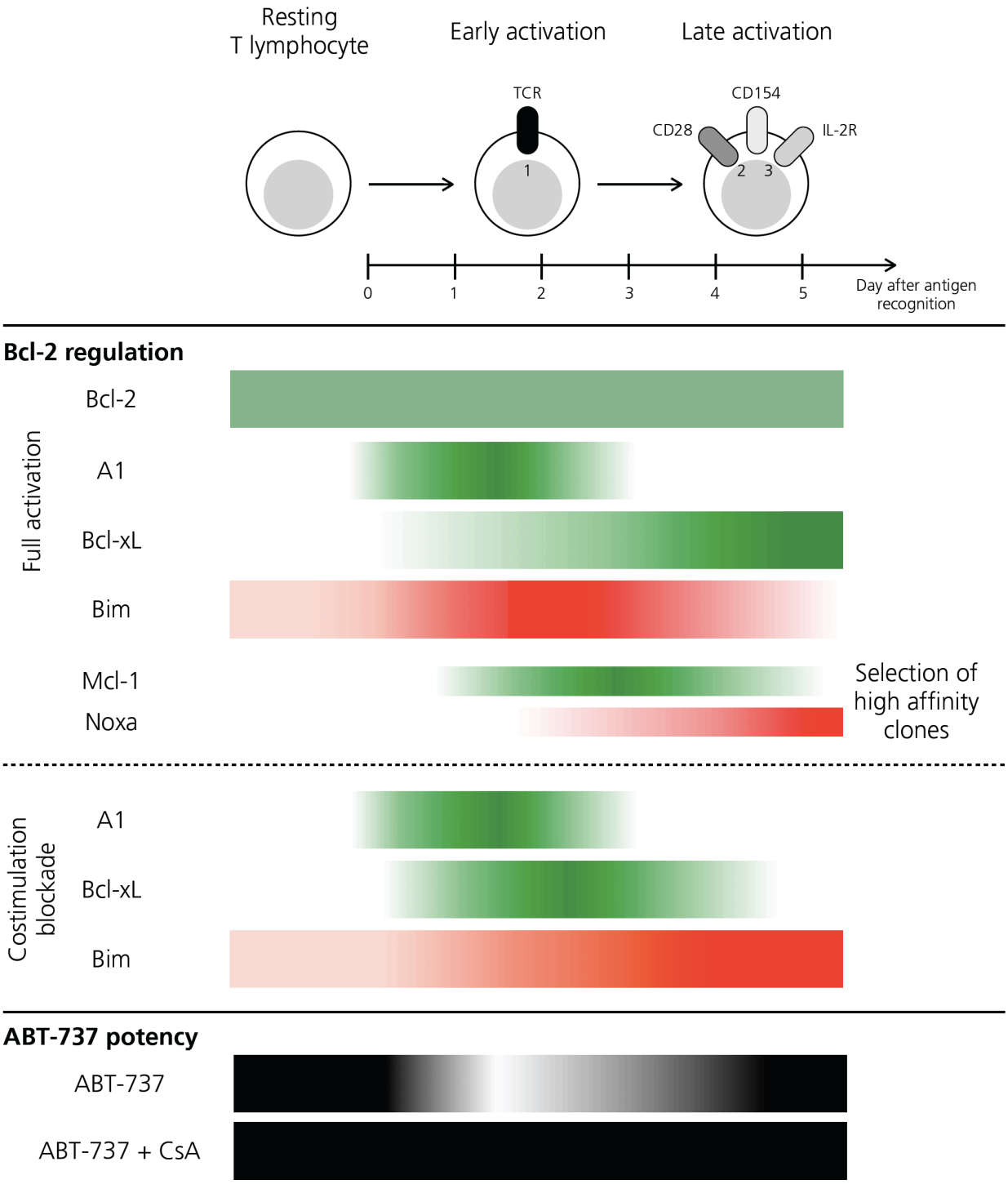

Fig. 34. Simplified model of Bcl-2 regulation in activated T cells

In the early phase after antigen recognition (about 2 days) signal 1 induces an up-regulation of the anti-apoptotic A1 (Verschelde, Walzer et al. 2003) and the pro-apoptotic Bim (Sandalova, Wei et al. 2004). In the later phase (day 3-5 after antigen recognition) costimulation signals induce an up-regulation of Bcl-xL (Watts 2010) and a down-regulation of Bim (Sabbagh, Srokowski et al. 2006). In parallel, the regulation of Mcl-1 and Noxa interacts with these mechanisms and is involved in the competitive selection of T cell clones (Wensveen, van Gisbergen et al. 2010). Costimulation blockade inhibits the up-regulation of Bcl-xL and prevents the down-regulation of Bim leading to apoptosis of the T cell. These mechanisms have a major impact on the pro-apoptotic potency of ABT-737 during the course of an immune response.
Green: anti-apoptotic, red: pro-apoptotic.

not be achieved by ABT-737, but its depleting potency is not impaired by T cell activation in combination with CsA. These observations are important for the application of ABT-737 as an immunosuppressive and as a tolerogenic drug (s. chapter 8).
The pro-apoptotic potency of ABT-737 was not only modulated by T cell activation. We observed that also in steady-state conditions different T cell populations displayed a different sensitivity to ABT-737. Particularly interesting are preliminary data demonstrating that **Tregs** are partially resistant to ABT-737. If these data will be confirmed, Bcl-2 inhibitors may find a role for the development of new strategies to generate or select Tregs *in vitro* and *in vivo*.
The second important mechanism of action of Bcl-2 inhibitors is related to the critical role of the intrinsic apoptosis pathway in **peripheral lymphocyte selection**. This process appears to occur later after T cell activation, when signal 2 and 3 modulate the expression of pro- and anti-apoptotic Bcl-2 factors to delete auto-reactive T cells (Strasser, Puthalakath et al. 2008), to regulate the competitive selection of high affinity T cell clones (Wensveen, van Gisbergen et al. 2010) and to determine the contraction phase and the generation of memory cells at the end of the immune response (Wojciechowski, Tripathi et al. 2007). Bcl-2 inhibitors directly interact with these molecular processes, and our data strongly suggest that the tolerogenic effect of ABT-737 in combination with costimulation blockade is at least in part the consequence of a increased peripheral deletion of antigen-specific T cells. By boosting the role of Bim, ABT-737 shifts the balance between the anti-apoptotic (mainly Bcl-xL) and the pro-apoptotic factors (mainly Bim) towards the pro-apoptotic side promoting the elimination of reactive T cells in synergism with costimulation blockers. The impact of ABT-737 on **central T cell selection** remains to be evaluated.
The effect of ABT-737 on **B cells** was not directly investigated in this project. Analogous mechanisms as described here for the T cell compartment control B cell homeostasis (Marsden and Strasser 2003). Bcl-2, Bcl-xL and A1 are induced after B cell activation and the balance between Bim and Bcl-2 / Bcl-xL regulates the selection of B cells during the process of high affinity maturation in the germinal center (Kuss, Knodel et al. 1999; Do, Hatada et al. 2000; Smith, Light et al. 2000). In line with data showing an inhibition of plasma cell generation in mice immunized under ABT-737 (Carrington, Vikstrom et al. 2010), we observed a lower level of donor-specific antibodies in mice treated with ABT-737 after skin transplantation (Fig. 16). Memory B cells were significantly affected by ABT-737, whereas plasma cells in the BM seem to be more resistant to Bcl-2 inhibition (Carrington, Vikstrom et al. 2010). A better characterization of the effect of Bcl-2 inhibitors on the B cell compartment is required to exploit the immunomodulatory potential of these drugs in transplantation and tolerance (Kirk, Turgeon et al. 2010).
Additional mechanisms are likely to be involved in the immunomodulatory effect of Bcl-2 inhibitors. **Apoptotic cells** have tolerogenic properties, and the massive number of apoptotic cells observed in the lymphatic organs of mice treated with ABT-737 is likely to modulate the licensing of dendritic cells and may contribute to the tolerogenic effect of ABT-737 (Green, Ferguson et al. 2009). Moreover, Bcl-2 factors are increasingly recognized as important players in **cellular processes** not directly involved in apoptosis regulation, such as in T cell activation (Ludwinski, Sun et al. 2009). Bcl-2 inhibitors may disturb these mechanisms with different possible functional consequences.
In summary, the immunomodulatory effect of ABT-737 is **multi-factorial** and complex. The most important mechanisms of action of ABT-737 on the immune system are probably related to selection processes determined by the expression of pro- and anti-apoptotic factors during the course of the immune response and – in general – by different lymphocyte populations. As a result, a Bcl-2 inhibitor such as ABT-737 can deplete or select lymphocytes with the same antigen specificity depending on their differentiation

and activation state. To my knowledge, these properties have never been described for other drugs before. Although this project was focused on allogeneic immune responses, the same mechanisms are likely to generally occur in lymphocytes, so that Bcl-2 inhibitors can be considered as a new class of immunomodulatory agents with potentially broad experimental and clinical applications.

Potential applications

Because of its particular selectivity profile among lymphocytes, ABT-737 might assume immunosuppressive or immunostimulatory properties. This project was primarily focused on transplantation immunology, and a possible **stimulation** of the immune response using Bcl-2 inhibitors was not further investigated. However, the results obtained by a short course of ABT-737 treatment in the initial phase of the immune response suggest that resistance to ABT-737 in activated T cells can be used to select a population of antigen-specific T cells after antigen recognition. This phenomenon was detrimental for the application of ABT-737 as an immunosuppressive drug, but may be exploited for the generation and expansion of antigen-specific T cells *in vitro* and *in vivo*, with broad possible applications for tumor- and infection-immunology.

The multifactorial **immunosuppressive** and **tolerogenic** effect of ABT-737 might find multiple applications to control undesired immune responses. ABT-737 had a beneficial effect in different models of **autoimmunity** in mice (Bardwell, Gu et al. 2009). Interestingly, the immunosuppressive effect was evident if the treatment was started before inducing autoimmunity, but was very limited in a curative approach (Lawlor, Smith et al. 2011). This observation is perfectly in line with our mechanistic studies in allogeneic models, and I speculate that the efficacy of ABT-737 could be significantly increased in combination with low-dose CsA in most autoimmunity models. Similar results might be obtained in models for **chronic inflammatory bowel diseases**, currently in preclinical investigation (M. Hausmann, personal communication). Furthermore, a possible application of Bcl-2 inhibitors to inhibit **allergic reactions** has been proposed in consideration of the sensitivity of mast cells to ABT-737 (Karlberg, Ekoff et al. 2010). The relevance of our findings for **transplantation** is discussed in details in the next chapter.

Immunosuppression

Currently available immunosuppressive drugs are very effective to prevent and treat acute rejection, but minimally ameliorated the long-term outcome after solid organ transplantation, because of side effects and of their limited impact on chronic rejection (chapter 1). Therefore, the development of novel immunosuppressive strategies is surely necessary, but the introduction of new immunosuppressive drugs only makes sense if these will assure a **long-term efficacy** and a reduced **systemic toxicity**. In general, immunosuppressive drugs can be used for induction therapy, for maintenance immunosuppression or as a rescue therapy in case of rejection. The potential role of Bcl-2 inhibitors for these therpeutical modalities is discussed here.

Because activated T cells are resistant to ABT-737, this drug is surely not indicated for the **treatment of acute rejection** episodes. In contrast, because of the critical role of A1 in early-activated T cells, a selective **A1 inhibitor** may deplete alloreactive T cells and therefore inhibit acute rejection without leading to a generalized lymphopenia. The same approach may be indicated in the early phase after transplantation to prevent rejection or in combination with DST. These hypotheses need to be confirmed experimentally as soon as a selective A1 inhibitor will be available.

According to our observations in a skin graft model, ABT-737 may find a role for **maintenance immunosuppression**. Importantly, the immunosuppressive effect of ABT-737 was markedly increased in combination with low-dose CsA. The combination of low doses of immunosuppressive drugs with different pharmacological targets is generally considered a good option to reduce the overall toxicity and therefore improve long-term outcome. Bcl-2 inhibitors might represent a valid combination partner for immunosuppressive therapies with a **reduced CNI-exposure**. However, although ABT-737 and navitoclax displayed a favorable toxicity profile in the short-term, we have to be very cautious about their potential **side effects** in the long-term. A sustained lymphopenia is generally associated with an increased risk for infections and tumors, and although an increased thrombopoiesis may partially compensate the loss of platelets in the long-term, an increased risk for bleeding is probable. The effect of a long-term exposure to ABT-737 on tumor pathogenesis is controversial. An increased apoptosis rate is generally associated with an anti-neoplastic effect in the short term, but a compensatory hyper-proliferation may even lead to cancer in the affected tissues in the long term (Weber, Boger et al. 2010). Finally, particularly in combination with CsA, ABT-737 is likely to accelerate the process of hair graying (Cippa, Kamarachev et al., submitted).

In my opinion, **induction therapy** is the most promising application of ABT-737 in transplantation. A short course of ABT-737 before transplantation will be well tolerated by the patients and is likely to promote the process of "host-graft-adaptation" by reducing the clone size of allo-reactive T cells, by the generation of tolerogenic apoptotic bodies in lymphatic tissues and – probably – by selection of regulatory T cells. Compared to the currently available induction therapy, which is principally based on the injection of anti-CD25 or depleting antibodies, Bcl-2 inhibitors are likely to induce a better depletion of memory cells and may find an application in sensitized patients. Moreover, **lymphopenia** after ABT-737 treatment recovers during a few days after treatment discontinuation whereas a single injection of ATG leads to a sustained lymphopenia over months. Therefore, induction by Bcl-2 inhibition may be favorable because it allows the physician to control the treatment depending on the clinical situation and for example to reduce the treatment duration in case of life-threatening infections. Furthermore, the

application of a **small molecule** such as ABT-737 is usually better tolerated and is associated with fewer risks compared with antibody therapy.
To take full advantage of all the immunomodulatory properties of ABT-737 the induction therapy should be combined with **DST** and **costimulation blockade**. In our model, the combination of a short course of ABT-737, MR1 and DST induced **donor-specific hyporesponsiveness**, as shown by a dramatic skin graft survival prolongation, and allowed to completely overcome the barrier to costimulation blockade provided by memory cells. This approach may find a near-term clinical application as an induction therapy followed by standard immunosuppression, but can also represent the basis for the development of tolerance induction protocols.

Tolerance induction
Tolerance induction is often considered as the holy grail of transplantation. Despite the major advantages achieved in the last decades, translation of established experimental models to primates and patients has not been uniformly successful. I propose a "**three compartment model**" to explain the disparities encountered in different animal models and to characterize the potential role of ABT-737 as a tolerogenic drug (Fig. 35).
The induction of allospecific tolerance consists in a fundamental re-setting of the immune system with a re-definition of the immune self (chapter 1). This process depends on the tolerization of three compartments of the adaptive immune system: (1) newly-arising lymphocytes, (2) naïve lymphocytes and (3) memory lymphocytes. "Tolerization" has to be considered as a combination of **deletion** and **regulatory mechanisms** that leads to the elimination or to the functional inactivation of allo-reactive T cells. The history of tolerance induction – from the seminal experiment of Medawar in mouse embryos, through the first mixed chimerism induction protocols in mice until the recent clinical applications – nicely reflects the disparities in these compartments in different experimental models (chapter 1). The induction of tolerance in mouse embryos only requires the tolerization of the first compartment and can be achieved without a manipulation of the peripheral immune system. In naïve laboratory mice, young animals living in a pathogen-free environment, induction and maintenance of tolerance is possible if the first and second compartment are successfully tolerized. Finally, the tolerization of memory cells is crucial in large animals, non-human primates and in patients. Thus, a clinically applicable tolerance induction protocol consists of an **induction** phase targeting the second and the third compartment and of a **maintenance** phase controlling the first compartment.
Extensive investigations about the processes involved in the tolerization of **peripheral naïve T cells** showed that regulatory mechanisms are not sufficient to induce tolerance across MHC-mismatched barriers in non-immunocompromised animals (Lechler, Garden et al. 2003). Deletion is the fundamental mechanism of tolerance during the induction phase. The most radical way to eliminate alloreactive T cells in mice is a complete T cell depletion by irradiation or depleting antibodies (Ildstad and Sachs 1984). An important step in the development of tolerance induction was the discovery of **costimulation blockers**. These induce a selective deletion of alloreactive T cells primarily by activation of the intrinsic apoptosis pathway (Wekerle, Kurtz et al. 2002). The prerequisites to obtain a generalized deletion of allo-reactive T cells by costimulation blockade are the activation of **signal 1** (Li, Li et al. 1999) and a previous reduction of the **clone size** (Ford, Wagener et al. 2008). Bcl-2 inhibitors are ideal pharmacological partners for costimulation blockers in this setting: ABT-737 induced clone size reduction and is likely to directly promote the process of peripheral deletion by activating the intrinsic apoptosis pathway. As a result, CsA even promoted the tolerogenic effect of ABT-737 in our model.
Memory T cells have more recently been recognized as a fundamental barrier for the translation of tolerance induction protocol

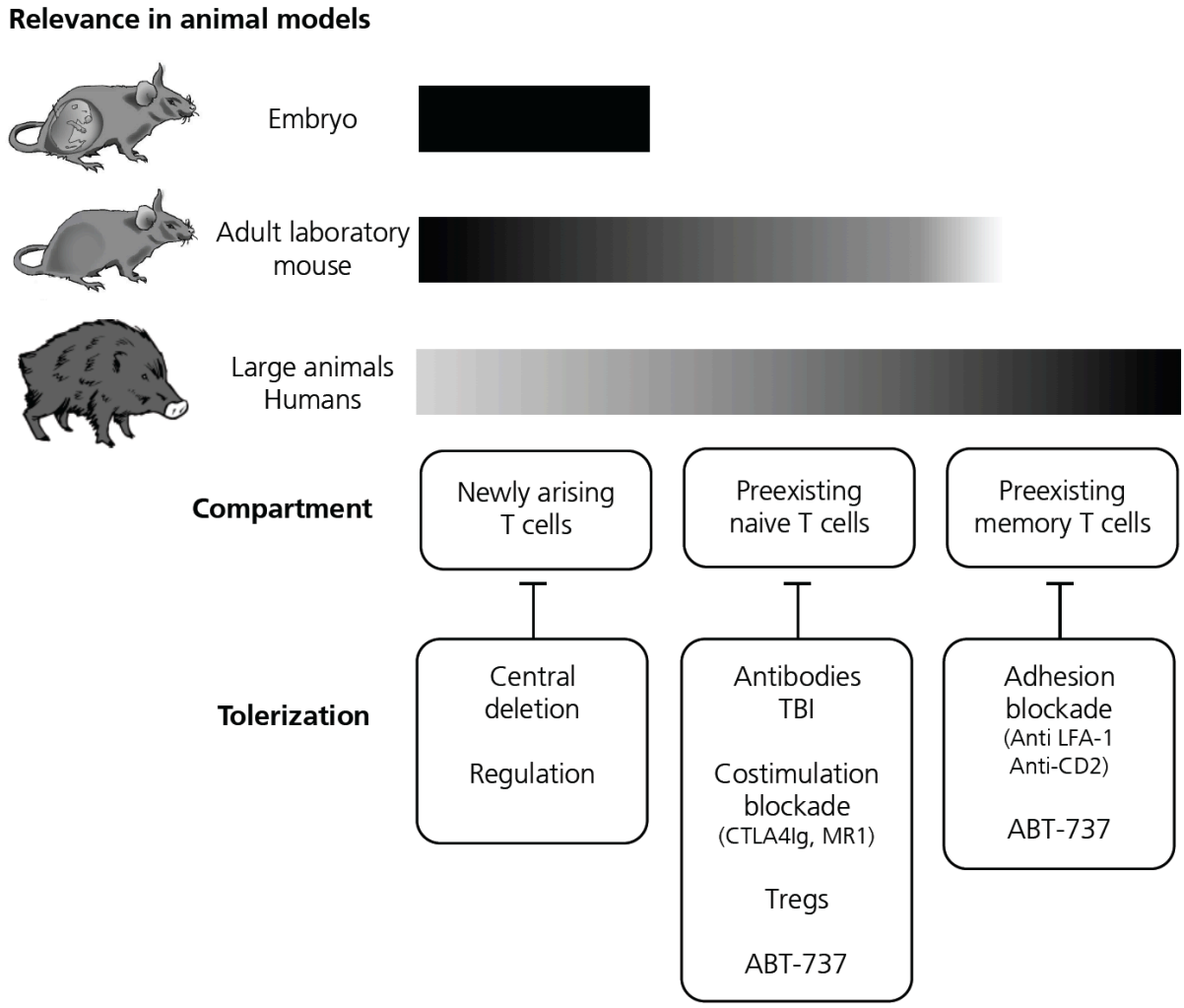

Fig. 35. A three compartment model for tolerance induction
According to the model described in the text, the three compartments, the theraputic options to tolerize them and their relevance in different animals models (black: high relevance, white: no relevance) are described.

to primates (Ford and Larsen 2011). Memory cells are almost absent in the immune system of laboratory animals, but progressively replace naïve cells in humans in a process called **immune senescence** as a result of the daily exposure to pathogens and of the progressive functional decline of the thymus (McElhaney and Effros 2009). Importantly, compared to naïve T cells, memory cells are much more resistant to costimulation blockade (Adams, Williams et al. 2003), depletional therapy (Pearl, Parris et al. 2005) and regulation (Yang, Brook et al. 2007), and - despite major efforts in this field (Weaver, Charafeddine et al. 2009) – efficacious options to inhibit memory cells are still not available. This limits the therapeutic options not only for sensitized patients, but – as a consequence of **heterologous immunity** – also the general efficacy of tolerance induction protocols based on costimulation blockade (Adams, Williams et al. 2003). A recent study demonstrated that the success of tolerance induction in non-human primates was associated to the number of preexisting donor-reactive memory T cells, indicating that **quantitative and qualitative** parameters may be relevant in this setting (Nadazdin, Boskovic et al. 2011). Therefore, a partial deletion of donor-reactive memory

cells may be sufficient to restore sensitivity to costimulation blockade and allow the induction of tolerance without completely impairing the immune memory against pathogens. ABT-737 efficiently depleted memory cells and restored sensitivity to costimulation blockade in a skin graft model with DST. If the same effect will be confirmed in a tolerance induction model and in large animals, Bcl-2 inhibitors may play a fundamental role for the translation of the mixed chimerism model to the clinic.

To maintain tolerance after a successful induction phase, it is necessary to control the generation and the activation of **newly arising T cells** that were not exposed to the tolerization process. As a result, a complete peripheral deletion of alloreactive T cells was only sufficient to induce tolerance after skin transplantion in thymectomized mice (Khan, Tomita et al. 1996). Regulatory or deletional mechanisms can be exploited to tolerize newly arising T cells: in mixed chimerism this is achieved by a central deletion of alloreactive thymocytes, whereas the generation of regulatory T cells – often induced by the graft itself – is pivotal in other models. The relevance of this compartment in the clinical setting remains a matter of debate: because of the reduced activity of the thymus in adults, it is not clear if strong immunological mechanisms are required to control the minimal number of newly arising lymphocytes or if weaker intra-graft regulation processes may be sufficient to maintain tolerance after graft adaptation. This may explain the controversial results of the first pilot studies using the mixed chimerism approach in patients (Kawai, Cosimi et al. 2008). Although all these patients lost chimerism during the first weeks after transplantation, the kidney graft was not rejected. This phenomenon, called "**split tolerance**", supports the hypothesis that intragraft mechanisms may maintain tolerance, but the underlying immunological mechanisms are still not completely understood. Similar mechanisms may explain how an induction therapy with ABT-737 as a single agent induced long-term survival of minimally immunogenic pancreas islet transplanted under the kidney capsule (Carrington, Vikstrom et al. 2010). In our fully MHC mismatched model, ABT-737 in combination with CsA and MR1 allowed the engraftment of bone marrow and induced a stable, low-level chimerism leading to systemic tolerance. Further experiments are required to assess the effect of ABT-737 on Tregs and on central selection, particularly on the thymus engraftment of donor-derived antigen presenting cells after bone marrow transplantation.

Clinical relevance and experimental outlook

In a clinical perspective, the most promising results obtained in this study are related to the tolerogenic effect of ABT-737 and to its efficacy on memory cells. The experience from clinical trials in oncology will show if the **toxicity profile** of navitoclax would allow a long-term therapy in patients with a non-malignant disease. In any case, available data strongly suggest that a short therapy with navitoclax should be associated with a favorable toxicity, so that the further development of our approach towards a clinical application seems reasonable. Particularly because of the effect on memory cells ABT-737 might represent an important factor for the development of the mixed chimerism approach towards a broad clinical application. Therefore, **tolerance** should be considered as the final goal, but a multi-step procedure is necessary for a gradual introduction of Bcl-2 inhibitors in transplantation without harm for the patients. Moreover, because of the excellent results achieved with conventional immunosuppressive drugs in current clinical use, for ethical reasons, the establishment of a tolerogenic therapy has to be developed in combination with a standard immunosuppression. In this context, the opportunity to prevent the anti-tolerogenic effect of CNIs using ABT-737 is of fundamental importance. Thus, the approach established in this project should be further developed on three parallel tracks in the clinic, in animal models and at the bench.

A multi-step approach to introduce ABT-737 in **clinical transplantation** should be considered. First, the efficacy of ABT-737 and navitoclax as immunosuppressive drugs and particularly on memory cells has to be confirmed on **human cells** *in vitro* before proceeding to the first interventional studies in patients. Thereafter, the less dangerous way to initiate a clinical application of navitoclax in transplant recipients is probably an induction therapy in highly **sensitized patients**. According to our data, the effect of navitoclax in combination with DST and costimulation blockade should lead to a depletion of donor-reactive memory cells and reduce the reactivity of the recipient towards donor cells. Thus, the efficacy of navitoclax *in vivo* may be first assessed comparing the *in vitro* reactivity of recipient cells towards donor cells before and after treatment with DST and navitoclax, ideally in combination with costimulation blockade. Although this is a hard immunological challenge, if successful this procedure would allow the patient to proceed to transplantation, whereas a negative result would not worsen the clinical situation. Only if the *in vitro* results will be successful, we could then proceed to a protocol primarily finalized at organ transplantation in sensitized and non-sensitized recipients. This would represent the first step towards an **induction therapy** based on Bcl-2 inhibition. One major limitation for the clinical application of this approach is related to the fact that anti-CD154 antibodies were withdrawn from the market because of thromboembolic side effects (Kawai, Andrews et al. 2000). CTLA4Ig has recently been introduced in the clinic, but was not sufficient to substitute MR1 in our model. The recent development of anti-CD40 blocking agents in our lab and by others may represent a good option in this setting (Page, Srinivasan et al. 2012).
In parallel, our **tolerance** induction protocol has to be further developed in animal models. Particularly, its application in **large animals** is absolutely required. Moreover, several practical aspects should be further investigated in the **mouse**. Additional experiments are required to minimize the conditioning phase, trying for example to reduce the exposure to ABT-737 to the pre-operative phase. Furthermore, a model to investigate our tolerance induction protocol in sensitized mice would be very informative. The presence of pre-existing donor-specific antibodies precluded the induction of tolerance in our mouse model, but this problem is manageable in the clinical setting, where therapies targeting the B cells and plasmapheresis are available. Another pratical issue is related to the timing of tolerance induction. The group of David Sachs recently showed in primates that the tolerance induction protocol is also effective if performed months after transplantation in recipients previously treated with standard immunosuppression (Yamada, Boskovic et al. 2011). This would represent a great advantage in the clinic. The synergistic effect of ABT-737 and CsA may be beneficial in this setting.
Finally, further **mechanistic studies** are necessary to better understand the regulation of apoptosis in the context of transplantation and tolerance in order to completely exploit the potential of this approach. The exact mechanism leading to CD8 T cell apoptosis under the effect of anti-CD154 is still incompletely understood, but it is very unlikely that a direct signal through CD154 provides a survival stimulus to CD8 T cells. Further studies are required to assess which signal is directly involved in the regulation of the intrinsic apoptosis pathway, and particularly in the regulation of Bim, in alloreactive T cells under the effect of costimulation blockade. The relative resistance to ABT-737 observed in Tregs and the effect of ABT-737 on thymus engraftment and on central selection is of critical interest for the further development of this approach.

The fascinating complexity of nature is an insurmountable obstacle for a complete understanding of the "immune self". However, a pragmatic application of our minimal knowledge may result in a substantial benefit for the quality of life of our patients. Memory cells and the toxicity of conditioning regimens are currently considered the

most important barriers to tolerance induction as an ideal strategy to prevent solid allograft rejection. The future will tell us, if a pharmacological modulation of the apoptosis pathway in lymphocytes might represent a good solution to these problems.

"Considerate la vostra semenza:
fatti non foste a viver come bruti,
ma per seguir virtute e canoscenza".

(Dante Alighieri, Divina Commedia,
Inferno, Canto XXVI)

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